UNIVERSIDADE FEDERAL DE VIÇOSA

MAYARA LOSS FRANZIN

POTENTIAL OF *Metarhizium* FROM SOILS AND RHIZOSPHERE OF DIVERSIFIED COFFEE SYSTEMS IN INSECT SUPPRESSION AND ITS INDIRECT EFFECT ON A COFFEE PEST THROUGH ENDOPHYTIC ASSOCIATION

VIÇOSA - MINAS GERAIS
2021

MAYARA LOSS FRANZIN

POTENCIAL DO *Metarhizium* PRESENTE NO SOLO E RIZOSFERA DE SISTEMA DE CAFÉ DIVERSIFICADO NA SUPRESSÃO DE INSETOS E SEU EFEITO INDIRETO SOBRE PRAGA DO CAFÉ ATRAVÉS DA ASSOCIAÇÃO ENDOFÍTICA

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

Orientadora: Madelaine Venzon

Coorientadores: Simon Luke Elliot
Marcos Antonio Matiello Fadini
Camila Costa Moreira

VIÇOSA - MINAS GERAIS
2021

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
F837p
2021

Franzin, Mayara Loss, 1992-
Potential of *Metarhizium* from soils and rhizosphere of diversified coffee systems in insect suppression and its indirect effect on a coffee pest through endophytic association / Mayara Loss Franzin. – Viçosa, MG, 2021.
1 tese eletrônica (124 f.): il. (algumas color.).

Inclui apêndice.
Orientador: Madelaine Venzon.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Entomologia, 2021.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2021.171
Modo de acesso: World Wide Web.

1. Fungos entomopatogênicos. 2. Fungos endofíticos. 3. Fungos do solo. 4. Coffea arábica. 5. Café - Doenças e pragas - Controle biológico. I. Venzon, Madelaine. II. Universidade Federal de Viçosa. Departamento de Entomologia. Programa de Pós-Graduação em Entomologia. III. Título.

CDD 22. ed. 632.46

Bibliotecário(a) responsável: Alice Regina Pinto CRB6 2523

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APROVADA: 18 de outubro de 2021.

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Mayara Loss Franzin
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Aos meus pais Nilda e Ademir, minhas irmãs Jussara e Viviam, minhas sobrinhas Gabrielly, Maria Eduarda e Ana Júlia, meu esposo Paulo Henrique, os maiores amores da minha vida.

DEDICO

AGRADECIMENTOS

Ao Programa de Pós-Graduação em Entomologia e a Universidade Federal de Viçosa por toda estrutura e suporte para a realização dos meus estudos.

A todos os professores Programa de Pós-Graduação em Entomologia pelas aulas e bate-papo que me concederam muito aprendizado nesses quatro anos de Doutorado.

À Empresa de Pesquisa Agropecuária de Minas Gerais -EPAMIG- Unidade Regional Zona da Mata e Fazenda Experimental de Patrocínio, por toda estrutura utilizada nos experimentos.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa.

À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Consórcio Brasileiro de Pesquisa e Desenvolvimento do Café (CBP & D-Café) pelo apoio na realização deste projeto.

À minha orientadora Madelaine Venzon, nossa eterna Fada Sensata do Controle Biológico, por ter aceitado me orientar. Pelo lindo trabalho que desenvolvemos nesta tese, do qual eu sinto um orgulho imenso. Por ser exemplo de profissional e pessoa. Por ser fonte de inspiração para mim. Por orientar com um olhar tão humano, sempre prezando não só pelo crescimento profissional dos seus alunos, mas também por nossa saúde física e mental. Pela orientação, conselhos, amizade, risadas, viagens e drinks.

Ao meu coorientador Sam, por toda orientação, por estar sempre disposto a me ouvir e colaborar com o meu trabalho. Por ter aberto as portas do Laboratório de Interações Inseto-Microrganismos e ter me deixado "se sentir em casa".

Ao meu coorientador Marcos Antonio Matiello Fadini por todo suporte com as análises estatísticas. Pelas inúmeras reuniões on-line para discutirmos sobre estatística e escrita. Por ser exemplo de disciplina e organização desde o meu mestrado.

À minha coorientadora Camila Costa Moreira, pela orientação sobre o mundo da microbiologia.

Aos membros da banca Dra. Wânia Neves, Dra. Camila Moreira, Dr. Angelo Pallini e Dr. Robert Barreto pela disponibilidade em compor a banca de avaliação desta tese.

Ao laboratório de Interações Inseto-Microrganismos, em especial Keminy, Marcela, Elenir, Walysson, Eduardo, Leila e Verônica, pelo suporte com os protocolos de meio de cultura, por facilitar o meu acesso ao laboratório e pela agradável companhia.

À Wânia dos Santos Neves pela ajuda na identificação dos fungos. Muito obrigada pelo suporte no preparo de lâminas e por toda paciência.

Ao funcionário Mario do viveiro de café por disponibilizar as mudas de café utilizadas no experimento do capítulo 3.

À estagiária Leila Nascimento pela ajuda imensurável na realização dos experimentos do capítulo 3.

À colega Adriana Wallerius pela ajuda na busca por bicho-mineiro para começar a criação no laboratório.

A todos que dividiram o Laboratório de Entomologia da Epamig comigo nesses quatros anos Elem, Jéssica Mayara, Jéssica Martins, Fernanda, Juliana Martinez, Juliana Maria, Gabriel Pantoja, Tércio, Letícia, Thais, Caro, Álvaro, João, Jéssily, Gabriel Pio, Gustavo, Mathias, André, Douglas e Pedro pelo agradável convívio e toda ajuda nos experimentos.

Aos funcionários da Epamig Sudeste, em especial Wânia, Marcelo, Rita, Geraldinho, Canuto, Cidinha, Maira e Miguel, pelo convívio diário, pelas muitas risadas e pelo crescimento pessoal e profissional.

Aos funcionários da Fazenda Experimental da Epamig de Patrocínio, Jaime, Diego, Greice, Nenzinho, Juliana, Marcela, Ronaldo, Hélio e os safristas que deram um apoio fundamental na condução da área experimental e na realização das nossas coletas e avaliações.

À Jéssica Mayara que divide a caminhada da vida comigo desde o ensino médio, pela ajuda nos experimentos, pela amizade, cumplicidade, respeito e parceria. Esse doutorado é mais um fruto (e o mais bonito) da nossa parceria.

As amizades vieram de outras instituições e que tive o privilégio de reencontrar no doutorado, Fernanda, Jéssica Martins e Wânia, pela amizade e ajuda nos experimentos.

A Elem, que é um dos maiores presentes que Viçosa me concedeu, pela ajuda nos experimentos pela amizade, pelas risadas de perder o fôlego e pelo ombro amigo nos momentos tristes. Jéssica Mayara, você e eu formamos um trio incrível!

A toda minha família: meus pais Nilda e Ademir, minhas irmãs Jussara e Viviam, minhas sobrinhas Gaby, Maria e Júlia, meu cunhado Abílio, minha avó Mariota e meus dois anjos da guarda que se foram no decorrer do Doutorado Adamo e Olga. Obrigada pelo apoio, torcida, carinho e por acreditarem mais em mim do que eu mesma. Amo vocês!

Ao meu esposo Paulo Henrique pelo apoio, amizade, carinho, amor e respeito. Por ter me levantado todas as vezes que estava desanimada e por sempre me ajudar a acreditar que sou capaz. Te amo!

A Deus, autor e dono da minha vida, que me guia pela vida sempre me concedendo a alegria de conviver com pessoas tão especiais como estas citadas acima!

“Tudo posso naquele que me fortalece.”

(Filipenses, 4:13)

RESUMO

FRANZIN, Mayara Loss, D.Sc., Universidade Federal de Viçosa, July, 2021. **Potencial do *Metarhizium* presente no solo e rizosfera de sistema de café diversificado na supressão de insetos e seu efeito indireto sobre praga do café através da associação endofítica.** Orientadora: Madelaine Venzon. Coorientadores: Simon Luke Elliot, Marcos Antonio Matiello Fadini e Camila Costa Moreira.

Sistemas de cultivo diversificados potencializam o serviço ecossistêmico de controle biológico de pragas, via atração e manutenção de inimigos naturais e melhoram as características químicas e biológicas do solo. Os fungos entomopatogênicos no solo, como o gênero *Metarhizium*, também são favorecidos pelos sistemas diversificados, por promoverem solos com menor incidência solar direta e maior umidade. Os fungos deste gênero são importantes agentes de controle microbiano de insetos-praga, o que os torna uma importante ferramenta para o manejo de pragas. Além disso, este gênero também é considerado como endofítico, colonizando raízes de plantas e podendo atuar no crescimento vegetal e na proteção indireta contra pragas. Neste trabalho, implantamos no Cerrado de Minas Gerais, parcelas de café diversificado, através da associação de plantas que atraem e mantém inimigos naturais, e investigamos o efeito dessa diversificação na conservação de *Metarhizium* spp. no solo. O café foi associado a árvores de *Inga edulis* (Fabaceae), *Senna macranthera* (Fabaceae) e a arbustos perenes de *Varronia curassavica* (Cordiaceae). Plantas espontâneas foram mantidas entre as linhas de café, por fornecerem recursos alimentares para predadores e parasitoides, exceto durante a colheita. Como parcelas controle utilizamos monocultivo de café convencional, com utilização de pesticidas. No Capítulo 1, avaliamos a atividade e a densidade de *Metarhizium* no solo, as características químicas e a atividade enzimática do solo dos sistemas diversificado e convencional, ao longo do tempo. Além disso, avaliamos a produção do café nos dois sistemas através do volume em litros de frutos produzidos por planta e o peso médio de 100 frutos. A avaliação da produção foi feita a partir da colheita de 2020. A atividade e densidade de *Metarhizium* spp. foram avaliadas usando a técnica de sobrevivência do inseto-isca *Tenebrio molitor* (Coleoptera: Tenebrionidae) e plaqueamento de suspensões de solo em meio de cultura seletivo sólido para contagem de unidades formadoras de colônias (UFC), respectivamente. Doze meses após o início do experimento, os insetos-isca morreram duas vezes mais rápido no sistema diversificado do que no convencional, além da densidade de *Metarhizium* spp. também ter sido maior. A atividade da enzima beta-glicosidase foi maior no sistema de café diversificado, indicando maior decomposição da matéria orgânica e ciclo do carbono neste

sistema. Além disso, não houve diferença de volume de frutos por plantas entre os dois sistemas de café, porém, em 2021, mas o peso de 100 frutos foi maior no sistema diversificado. No Capítulo 2, foi investigada a associação de *Metarhizium* spp. à rizosfera das plantas dos sistemas de café diversificado e convencional. Foram usados os mesmos protocolos do Capítulo 1 para avaliar a atividade e densidade de *Metarhizium*. A atividade e a densidade de *Metarhizium* spp. em amostras de solo e raízes foram maiores no sistema de café diversificado do que no convencional. *Metarhizium* spp. foi isolado das raízes de *I. edulis, V. Curassavica, S. macranthera, Gnaphalium spicatum, Conyza bonariensis, Solanum Americanum, Ageratum conyzoides, Bidens pilosa, Sida cordifolia* e café no sistema diversificado. No Capítulo 3, investigamos se *Metarhizium robertsii* e *Metarhizium brunneum*, isolados de raízes de café do sistema de café diversificado no Cerrado, afetam o crescimento de mudas de café e seu efeito sobre o ataque de bicho-mineiro do café *Leucoptera coffeella* (Lepidoptera: Lyonetiidae). *Metarhizium* spp. foi isolado da maioria das raízes das mudas de café inoculadas, indicando que esses fungos podem colonizar as raízes do café via inoculação no substrato. Foi constatado que *M. robertsii* aumentou a área foliar das mudas de café. Além disso, as mudas de café que inoculadas com os dois isolados de *Metarhizium* tiveram menor porcentagem de área foliar minada e o tempo de desenvolvimento do bicho-mineiro foi maior nessas plantas. Portanto, nesta tese foi elucidado que a diversificação estratégica de cultivos de café aumenta a densidade e atividade de *Metarhizium* no solo ao longo do tempo, aumentando a produção de café. Além disso, as espécies *M. brunneum* e *M. robertsii* apresentam potencial para serem utilizadas como inoculantes de mudas de café, uma vez que as mudas onde estes foram inoculados apresentaram menores danos causados por bicho-mineiro. Portanto, a diversificação estratégica de cultivos de café pode ser uma alternativa para os monocultivos, melhorando a qualidade do solo e a produtividade do café.

Palavras-chave: Cultivo diversificado. Café arábica. Fungo entomopatogênico. Fungo endofítico. Solo supressivo.

ABSTRACT

FRANZIN, Mayara Loss, D.Sc., Universidade Federal de Viçosa, July, 2021. **Potential of *Metarhizium* from soils and rhizosphere of diversified coffee systems in insect suppression and its indirect effect on a coffee pest through endophytic association.** Adviser: Madelaine Venzon. Co-advisers: Simon Luke Elliot, Marcos Antonio Matiello Fadini and Camila Costa Moreira.

Diversified crop systems enhance ecosystem services, such as pest biological control, via attraction and maintenance of entomophagous, and improve soil chemical and biological characteristics. Soil entomopathogenic fungi, such as *Metarhizium* spp*.,* are also benefit in diversified systems, because these systems reduce direct soil irradiation and increase soil humid. The fungi of this genera are important microbial control agents of insect pests, which made them an important tool to pest management. Besides, this genus is also considered as endophytic, colonizing roots of plants, increasing plant growth and promoting protection against pests. Here, we designed a strategic diversified coffee system in the Cerrado of Minas Gerais, by the associating coffee with plants that attract natural enemies, and investigated the effects of such diversification in the *Metarhizium* conservation in soil. Coffee was associated with trees of *Inga edulis* (Fabaceae) and *Senna macranthera* (Fabaceae) and perennial bushes of *Varronia curassavica* (Cordiaceae). Non-crop plants were maintained between coffee rows, except before the coffee harvesting, as they can provide food resources for predators and parasitoids. As control, we designed plots with conventional coffee monoculture, with the standard conventional use of pesticides. In Chapter 1, we evaluated *Metarhizium* activity and density, soil chemical characteristics and enzymatic activity from diversified and conventional soil of coffee systems over time. We estimated the coffee yield in both coffee systems by measuring the volume of coffee fruits per plant and the weight of 100 fruits. The activity and density of *Metarhizium* spp. was assessed using the bait survival technique with *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae as bait, and plating soil suspensions on to solid selective media for counts of colony-forming units (CFU), respectively. Twelve months after experiment starts, bait insects died two times faster due to *Metarhizium* spp. in the diversified than in the conventional coffee systems, while the density of *Metarhizium* spp. was also higher in the diversified. Beta-glycosidase activity was greater in the diversified coffee system, indicating more organic matter decomposition and carbon cycle in this coffee system. Furthermore, there was no difference of volume of fruit between coffee systems, however, the weight of 100 fruits was higher in diversified system in 2021. In Chapter 2, we investigated

Metarhizium spp. rhizosphere association with plant roots from diversified and conventional coffee system. We used the same protocols of Chapter 1 to evaluated *Metarhizium* activity and density. Both were higher in soil and roots samples from diversified than conventional system. We isolated *Metarhizium* spp. from *I. edulis, V. Curassavica, S. macranthera, Gnaphalium spicatum, Conyza bonariensis, Solanum Americanum, Ageratum conyzoides, Bidens pilosa, Sida cordifolia* and coffee plants of diversified coffee system. In Chapter 3, we investigate whether *Metarhizium robertsii* and *Metarhizium brunneum,* isolated from coffee roots from diversified coffee system in Cerrado*,* improve coffee seedlings growth and indirectly protect them against the coffee leaf miner (CLM) *Leucoptera coffeella* (Lepidoptera: Lyonetiidae). We recovered *Metarhizium* spp. from most of coffee seedling roots, indicating that these fungi can colonize coffee roots by soil drench. We found that *M. robertsii* increased the leaf area of coffee seedlings. Besides, the plants inoculated with *Metarhizium* isolates had lower percentage of mined leaf area and the development time of CLM was higher in these plants. Therefore, in this thesis we showed that strategic plant diversification in coffee crops increases density and activity of *Metarhizium*, soil enzymatic activity and coffee yield over time. Besides, *M. brunneum* and *M. robertsii* have potential to use as inoculates of coffee seedlings, because the plants inoculated with them isolates showed lower CLM damage. Therefore, strategic plant diversification in coffee systems can be an alternative to monocultures, improving soil quality and coffee yield.

Keywords: Diversified crop. Arabica coffee. Entomopathogenic fungal. Endophytic fungal. Supressive soil

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SUMÁRIO

GENERAL INTRODUCTION

Hypocrealean entomopathogenic fungi are important microbial control agents of insect pests (Brunner-Mendoza et al., 2019; David, 1967; Jaramillo et al., 2015; Meyling and Eilenberg, 2007). The infection begins when hosts are exposed to the fungal conidia. They germinate by producing a germ tube and entry into the host body. Inside the host, the fungus multiplies, invades the host tissues, emerges from the host, and produces more conidia to continue the infection cycle (Alves, 1998). Fungal propagules can persist outside the host on soil and on phylloplane, which are reservoirs of these inoculum (Hesketh et al., 2010). Besides, soil is the habitat where much of the entomopathogenic fungi life cycle occurs, such as multiplication within hosts, saprophytic, endophytic and rhizosphere-competence growth (Elliot et al., 2000; Hesketh et al., 2010; Meyling and Eilenberg, 2007; Morgan et al., 2005; Pell et al., 2010). These fungi have been found to occur naturally in agricultural soils (Hernández-Domínguez et al., 2016; Kepler et al., 2015; Moreira et al., 2019; Sharma et al., 2018) and may play natural regulation of many arthropods that spend some or all of their time in the soil (Pell et al., 2010; Townsend et al., 1995).

The fungal genera *Metarhizium* (Hypocreales: Clavicipitaceae) is the most abundant entomopathogenic fungi in crop soils (Botelho et al., 2019; Hernández-Domínguez et al., 2016; Moreira et al., 2019; Roberts and St. Leger, 2004; Sharma et al., 2018). They infect arthropods (Brunner-Mendoza et al., 2019; David, 1967; Jaramillo et al., 2015; Meyling and Eilenberg, 2007) and also can colonize different plants (Behie et al., 2012; Behie and Bidochka, 2014; Fisher et al., 2011; Keyser et al., 2015; Krell et al., 2018). *Metarhizium* establishes mutualistic association with the host plant and colonizes their roots (Ahmad et al., 2020; Behie et al., 2015), stems and leaves (Ahmad et al., 2020; Batta, 2013), however, it is most common found in roots (Ahmad et al., 2020; Bamisile et al., 2018; Behie et al., 2012; Wyrebek et al., 2011). The association of *Metarhizium* with plant roots can be endophytic or rhizosphere-competent (Hu

and Leger, 2002) and both associations can promote plant growth and protection against pests (Behie et al., 2012; Behie and Bidochka, 2014; Jaber and Araj, 2018). Endophytic fungi colonize plant tissues without causing any symptoms of disease in the plant (Mantzoukas and Eliopoulos, 2020; Wilson, 1995). Rhizosphere-competent fungi did not colonize plant tissues internally, they grow in the rhizosphere (Morgan et al., 2005).

Due to *Metarhizium* association with plant roots, diversified agriculture systems with plants that they can associated, benefit *Metarhizium* conservation in soil (Liao et al., 2014; Nishi and Sato, 2019; St. Leger, 2008), which root exudates may stimulate fungi growth and activity (Baker, 1991; St. Leger, 2008). Besides, diversified crops systems generally have soil with high humidity and low solar radiation, which improve fungi processes such as sporulation, germination and infection of hosts (Driesche et al., 2008). Nevertheless, diversified agricultures systems are not common. Brazil is the largest producer of coffee in the world (FAOSTAT, 2019), where monocultures predominate and the use of pesticides is the most common control for coffee pests (Venzon, 2021). The municipality of Patrocínio, in the Cerrado of Minas Gerais region, is common extensive monoculture areas. This is the municipality that most produced coffee in Brazil, with 52,000 ha of plantation and a production approximately of 1,000,000 bags of green bean per year (Conab, 2021). Furthermore, monocultures commonly result in proliferation of pests and pathogens, with introduction of pesticides in intensively managed fields, which cause negative effects on soil biota (Brown et al., 2020), predators and parasitoids (Venzon, 2021).

Diversified coffee crops are known to mediate ecosystem services, such as biological control by entomophagous, via attraction and provision of food to natural enemies from associated plants (Rezende et al., 2014; Venzon, 2021). It is also known that they correlate positively with abundance and suppressive potential of insect pests by entomopathogenic fungi (Moreira et al., 2019). The soil quality, as physical characteristics and chemical composition,

and enzyme activities (Barrios, 2007), such as arylsulfatase, β-glucosidase and acid phosphatase (Mendes et al., 2018) are also higher on diversified systems than in full sun monocultures (Barrios et al., 2012; Rigal et al., 2020). Evaluations of enzyme activity are efficient indicators of microbiological activity, because the enzymes participate as catalysts in intracellular metabolic reactions that occur in soil microbiota involved in nutrient cycling (Ladd, 1985; Mendes et al., 2018; Miguel et al., 2020).

Thus, besides the attraction and provision of food to natural enemies (Rezende et al., 2014; Venzon, 2021), strategic plant diversification has the potential of improving soil quality (Barrios et al., 2012; Rigal et al., 2020), and conserving soil fungi, such as *Metarhizium* spp. (Moreira et al., 2019). All these soil characteristics are important to improve coffee yield, as it contributes to nutrient availability for the plants (Behie et al., 2012; Pavinato and Rosolem, 2008). Thereby, in this thesis we designed a strategic diversified coffee system without pesticides aiming to improve a conservation biological control of pests by predators, parasitoids, and *Metarhizium* spp. The latter is the object of our study. Ours system consisted of coffee associated with trees of *Inga edulis* (Fabaceae) (Rezende et al., 2014) and *Senna macranthera* (Fabaceae) (Marazzi et al., 2013, 2006) and perennial bushes of *Varronia curassavica* (Cordiaceae) (Venzon et al., 2018). Non-crop plants were maintained between coffee rows, as they can provide food resources for predators and parasitoids (Amaral et al., 2013; Venzon et al., 2019). We compared this system to conventional coffee monoculture with the use of pesticides.

In Chapter 1, we evaluated over time *Metarhizium* activity and density, chemical characteristics and enzymatic activity from soil of diversified and of conventional coffee systems. Additionally, we measured the coffee yield in both coffee systems by the volume of coffee fruits per plant and the weight of fruits. We assessed *Metarhizium* activity by using the bait survival technique (Moreira et al., 2019) with *Tenebrio molitor* (Coleoptera:

Tenebrionidae) larvae. We also measured *Metarhizium* spp. density by plating soil suspensions on to selective media for counts of colony-forming units (CFU). Our objective was investigated whether strategic plant diversification in coffee crops can improve the activity and density of *Metarhizium* and coffee yield over time, besides chemical and enzymatic activity, that determine the quality of soils (FAO, 2020).

In Chapter 2, we investigated the rhizosphere association of *Metarhizium* spp. with plant roots from diversified and conventional coffee system. We used the same protocols of Chapter 1 to evaluated *Metarhizium* activity and density. Our objective was investigated whether *Metarhizium* spp. colonize plants roots of diversified coffee system, thereby improving *Metarhizium* spp. activity and density in this coffee system.

Based on *Metarhizium* spp. isolated in coffee roots of Chapter 2, we investigated in Chapter 3, whether *Metarhizium* spp. would improve the growth of coffee seedlings and would indirectly protect them from coffee leaf miner (CLM) *Leucoptera coffeella* (Lepidoptera: Lyonetiidae) damage. This pest causes damage to coffee plants since the nursery period, which reduces the seedlings quality through the reduction of leaf area and defoliation (Tomaz et al., 2015). We performed a greenhouse experiment with inoculation of *M. brunneum* and *M. robertsii* on coffee seedlings by soil drench. Subsequently, we infested the seedlings with CLM adults and evaluated the colonization of *Metarhizium* spp., the CLM development and the seedling growth.

Therefore, in this thesis we contributed to elucidate the benefits of strategic plant diversification of coffee crops in the Cerrado for *Metarhizium* soil conservation, soil quality and coffee yield, over time. Additionally, we showed the potential of *M. brunneum* and *M. robertsii* as inoculates of coffee seedlings.

References

Ahmad, I., Jiménez-Gasco, M.M., Luthe, D.S., Shakeel, S.N., Barbercheck, M.E., 2020. Endophytic *Metarhizium robertsii* promotes maize growth, suppresses insect growth, and alters plant defense gene expression. Biol. Control 144, 104167. https://doi.org/10.1016/j.biocontrol.2019.104167

Alves, S.B., 1998. Fungos entomopatogênicos, in: Alves, S.B. (Ed.), Controle Microbiano de Insetos. FEALQ, Piracicaba, pp. 289–381.

Amaral, D.S.S.L., Venzon, M., Duarte, M.V.A., Sousa, F.F., Pallini, A., Harwood, J.D., 2013. Non-crop vegetation associated with chili pepper agroecosystems promote the abundance and survival of aphid predators. Biol. Control 64, 338–346. https://doi.org/10.1016/j.biocontrol.2012.12.006

Baker, R., 1991. Diversity in biological control. Crop Prot. 10, 85–94. https://doi.org/10.1016/0261-2194(91)90054-U

Bamisile, B.S., Dash, C.K., Akutse, K.S., Keppanan, R., Wang, L., 2018. Fungal endophytes: Beyond herbivore management. Front. Microbiol. 9, 544. https://doi.org/10.3389/fmicb.2018.00544

Barrios, E., 2007. Soil biota, ecosystem services and land productivity. Ecol. Econ. 64, 269–285. https://doi.org/10.1016/j.ecolecon.2007.03.004

Barrios, E., Sileshi, G.W., Shepherd, K., Sinclair, F., 2012. Agroforestry and soil health: Linking trees, soil biota, and ecosystem services, in: Wall, D.H., Bardgett, R.D., Behan-Pelletier, V., Herrick, J.E., Jones, T.H., Ritz, K., Six, J., Strong, D.R., Putten, W. van der (Eds.), Soil Ecology and Ecosystem Services. Oxford University Press, Oxford UK, pp. 315–330.

Batta, Y.A., 2013. Efficacy of endophytic and applied *Metarhizium anisopliae* (Metch.) Sorokin (Ascomycota : Hypocreales) against larvae of *Plutella xylostella* L. (Yponomeutidae : Lepidoptera) infesting *Brassica napus* plants. Crop Prot. 44, 128–134. https://doi.org/10.1016/j.cropro.2012.11.001

Behie, S.W., Bidochka, M.J., 2014. Nutrient transfer in plant–fungal symbioses. Trends Plant Sci. 19, 734–740. https://doi.org/10.1016/j.tplants.2014.06.007

Behie, S.W., Jones, S.J., Bidochka, M.J., 2015. Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*. Fungal Ecol. 13, 112–119. https://doi.org/10.1016/j.funeco.2014.08.001

Behie, S.W., Zelisko, P.M., Bidochka, M.J., 2012. Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. Science. 336, 1576–1577. https://doi.org/10.1126/science.1222289

Botelho, A.B.R.Z., Alves-Pereira, A., Prado, R.C., Zucchi, M.I., Delalibera Júnior, I., 2019. *Metarhizium* species in soil from Brazilian biomes: a study of diversity, distribution, and association with natural and agricultural environments. Fungal Ecol. 41, 289–300. https://doi.org/10.1016/j.funeco.2019.07.004

Brown, G., Cooper, M., Kobayashi, M., 2020. Threats to soil biodiversity – global and regional trends, in: FAO, ITPS, GSBI, CBD, EC (Eds.), State of Knowledge of Soil Biodiversity: Status, Challenges and Potentialities. FAO, Roma, pp. 191–262.

Brunner-Mendoza, C., Reyes-Montes, M. del R., Moonjely, S., Bidochka, M.J., Toriello, C., 2019. A review on the genus *Metarhizium* as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico. Biocontrol Sci. Technol. 29, 83–102. https://doi.org/10.1080/09583157.2018.1531111

Conab, 2021. Acompanhamento da Safra Brasileira: Café. Companhia Nacional de Abastecimento. URL https://www.conab.gov.br/index.php/info-agro/safras/cafe (accessed 9.20.21).

David, W.A.L., 1967. The physiology of the insect integument in relation to the invasion of pathogens, in: Beament, J.W.L.; Treherne, J.E. (Ed.), Insects and Physiology. Edinburgh, &c., Oliver & Boyd., pp. 17–35.

Driesche, R. Van, Hoddle, M., Center, T., 2008. Control of pests and weeds by natural enemies: An introduction to biological control, 1st ed. Blackwell publishing Ltd, Oxford UK. https://doi.org/10.1653/024.092.0237

Elliot, S.L., Sabelis, M.W., Janssen, A., Van der Geest, L.P.S., Beerling, E.A.M., Fransen, J., 2000. Can plants use entomopathogens as bodyguards? Ecol. Lett. 3, 228–235. https://doi.org/10.1046/j.1461-0248.2000.00137.x

FAO, 2020. State of knowledge of soil biodiversity: Status, challenges and potentialities, 1st ed. FAO, Roma.

FAOSTAT, 2019. Food and Agriculture Organization of the United Nations. URL http://faostat.fao.org/faostat. (accessed 9.20.21).

Fisher, J.J., Rehner, S.A., Bruck, D.J., 2011. Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. J. Invertebr. Pathol. 106, 289–295. https://doi.org/10.1016/j.jip.2010.11.001

Hernández-Domínguez, C., Cerroblanco-Baxcajay, M. de L., Alvarado-Aragón, L.U., Hernández-López, G., Guzmán-Franco, A.W., 2016. Comparison of the relative efficacy of an insect baiting method and selective media for diversity studies of *Metarhizium* species in the soil. Biocontrol Sci. Technol. 26, 707–717.

https://doi.org/10.1080/09583157.2016.1152458

Hesketh, H., Roy, H.E., Eilenberg, J., Pell, J.K., Hails, R.S., 2010. Challenges in modelling complexity of fungal entomopathogens in semi-natural populations of insects. BioControl 55, 55–73. https://doi.org/10.1007/s10526-009-9249-2

Hu, G., Leger, R.J.S., 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. Appl. Environ. Microbiol. 68, 6383–6387. https://doi.org/10.1128/AEM.68.12.6383

Jaber, L.R., Araj, S.E., 2018. Interactions among endophytic fungal entomopathogens (Ascomycota: Hypocreales), the green peach aphid *Myzus persicae* Sulzer (Homoptera: Aphididae), and the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae). Biol. Control 116, 53–61. https://doi.org/10.1016/j.biocontrol.2017.04.005

Jaramillo, J.L., Montoya, E.C., Benavides, P., Góngora B, C.E., 2015. *Beauveria bassiana* y *Metarhizium anisopliae* para el control de broca del café en frutos del suelo. Rev. Colomb. Entomol. 41, 95–104.

Kepler, R.M., Ugine, T.A., Maul, J.E., Cavigelli, M.A., Rehner, S.A., 2015. Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. Environ. Microbiol. 17, 2791–2804. https://doi.org/10.1111/1462-2920.12778

Keyser, C.A., De Fine Licht, H.H., Steinwender, B.M., Meyling, N.V., 2015. Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. BMC Microbiol. 15, 249. https://doi.org/10.1186/s12866-015-0589-z

Krell, V., Jakobs-Schoenwandt, D., Vidal, S., Patel, A.V, 2018. Cellulase enhances endophytism of encapsulated *Metarhizium brunneum* in potato plants. Fungal Biol. 122,

373–378. https://doi.org/10.1016/j.funbio.2018.03.002

Ladd, J.N., 1985. Soil enzymes, in: Vaughan, D., Malcolm, R.E. (Eds.), Origin and range of enzymes in Soil. Developments in plant and soil sciences. Springer, Dordrecht, pp. 175–221.

Liao, X., O'Brien, T.R., Fang, W., St. Leger, R.J., 2014. The plant beneficial effects of *Metarhizium* species correlate with their association with roots. Appl. Microbiol. Biotechnol. 98, 7089–7096. https://doi.org/10.1007/s00253-014-5788-2

Mantzoukas, S., Eliopoulos, P.A., 2020. Endophytic entomopathogenic fungi: A valuable biological control tool against plant pests. Appl. Sci. 10, 360. https://doi.org/10.3390/app10010360

Marazzi, B., Conti, E., Sanderson, M.J., Mcmahon, M.M., Bronstein, J.L., 2013. Diversity and evolution of a trait mediating ant-plant interactions: Insights from extrafloral nectaries in *Senna* (Leguminosae). Ann. Bot. 111, 1263–1275. https://doi.org/10.1093/aob/mcs226

Marazzi, B., Fortunato, R.H., Endress, P.K., Spichiger, R., 2006. *Senna* (Cassiinae, Leguminosae) in Paraguay: Synopsis, occurrence, ecological role and ethnobotany. Candollea 61, 315–329.

Mendes, I.C., Sousa, D.M.G. de, Reis Junior, F.B. dos, Lopes, A.A. de C., 2018. Bioanálise de solo: como acessar e interpretar a saúde do solo. Circ. Técnica - Embrapa Cerrados 38, 23.

Meyling, N. V, Eilenberg, J., 2007. Ecology of the entomopathogenic fungi Beauveria bassiana and *Metarhizium anisopliae* in temperate agroecosystems : Potential for conservation biological control. Biol. Control 43, 145–155. https://doi.org/https://doi.org/10.1016/j.biocontrol.2007.07.007

Miguel, D.L., da Silva, E.M.R., da Silva, C.F., Pereira, M.G., Leite, L.F.C., 2020. Soil

microbiological properties and enzyme activity in agroforestry systems compared with monoculture, natural regeneration, and native caatinga. Biosci. J. 36, 1–16. https://doi.org/10.14393/BJ-v36n1a2020-42704

Moreira, C.C., Celestino, D., Guerra, T., Cardoso, I.M., Elliot, S.L., 2019. Agroforestry coffee soils increase the insect-suppressive potential offered by entomopathogenic fungi over full-sun soils : A case proposing a “bait survival technique.” Ecol. Evol. 9, 10777–10787. https://doi.org/10.1002/ece3.5598

Morgan, J.A.W., Bending, G.D., White, P.J., 2005. Biological costs and benefits to plant-microbe interactions in the rhizosphere. J. Exp. Bot. 56, 1729–1739. https://doi.org/10.1093/jxb/eri205

Nishi, O., Sato, H., 2019. Isolation of *Metarhizium* spp . from rhizosphere soils of wild plants reflects fungal diversity in soil but not plant specificity. Mycology 10, 22–31. https://doi.org/10.1080/21501203.2018.1524799

Pavinato, P.S., Rosolem, C.A., 2008. Disponibilidade de nutrientes no solo: decomposição e liberação de compostos orgânicos de resíduos vegetais. Rev. Bras. Ciênc. Solo 32, 911-920. https://doi.org/10.1590/S0100-06832008000300001

Pell, J.K., Hannam, J.J., Steinkraus, D.C., 2010. Conservation biological control using fungal entomopathogens. BioControl 55, 187–198. https://doi.org/10.1007/s10526-009-9245-6

Rezende, M.Q., Venzon, M., Perez, A.L., Cardoso, I.M., Janssen, A., 2014. Extrafloral nectaries of associated trees can enhance natural pest control. Agric. Ecosyst. Environ. 188, 198–203. https://doi.org/10.1016/j.agee.2014.02.024

Rigal, C., Xu, J., Vaast, P., 2020. Young shade trees improve soil quality in intensively managed coffee systems recently converted to agroforestry in Yunnan Province, China. Plant Soil 453, 119–137. https://doi.org/10.1007/s11104-019-04004-1

Roberts, D.W., St. Leger, R.J., 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. Adv. Appl. Microbiol. 54, 1–70. https://doi.org/10.1016/S0065-2164(04)54001-7

Sharma, L., Oliveira, I., Torres, L., Marques, G., 2018. Entomopathogenic fungi in portuguese vineyards soils: suggesting a 'Galleria-Tenebrio-bait method' as bait- insects *Galleria* and *Tenebrio* significantly underestimate the respective recoveries of *Metarhizium* (*robertsii*) and *Beauveria* (*bassiana*). MycoKeys 38, 1–23. https://doi.org/10.3897/mycokeys.38.26970

St. Leger, R.J., 2008. Studies on adaptations of *Metarhizium anisopliae* to life in the soil. J. Invertebr. Pathol. 98, 271–276. https://doi.org/10.1016/j.jip.2008.01.007

Tomaz, M.A., Martins, L.D., Rodrigues, W.N., 2015. Produção de mudas e plantio, in: Sakiyama, N.S., Martinez, H.E. Pietro, Tomaz, M.A., Borém, A. (Eds.), Café Arábica: Do Plantio à Colheita. UFV, Viçosa, pp. 46–63.

Townsend, R., Glare, T., Willoughby, B., 1995. The fungi *Beauveria* spp. cause epizootics in grass grub populations in Waikato., in: Proceedings of the 48th New Zealand Plant Protection Conference. New Zealand, pp. 237–241.

Venzon, M., 2021. Agro-ecological management of coffee pests in Brazil. Front. Sustain. Food Syst. 5, 721117. https://doi.org/10.3389/fsufs.2021.721117

Venzon, M., Togni, P.H.B., Chiguachi, J.A.M., Pantoja, G.M., Brito, E.A.S., Sujii, E.R., 2019. Agrobiodiversidade como estratégia de manejo de pragas. Inf. Agropecuário 40, 21–29.

Venzon, M., Togni, P.H.B., Amaral, D.S.S.L., Rezende, M.Q., Batista, M.C., Chiguachi, J.A.M., Martins, E.F., Perez, A.L., 2018. Mobilisation des mécanismes de régulation naturelle des ravageurs via des plantes à multiples services écosystémiques. Innov. Agron. 64, 83–95.

Wilson, D., 1995. Endophyte - the evolution of a term, and clarification of its use and definition. Oikos 73, 274–276.

Wyrebek, M., Huber, C., Sasan, R.K., Bidochka, M.J., 2011. Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. Microbiology 157, 2904–2911. https://doi.org/10.1099/mic.0.051102-0

Chapter I[1]

Strategic plant diversification in coffee crops increases density and activity of *Metarhizium* spp. in soil and improve coffee yield

Abstract

Strategic plant diversification may reduce ecosystem disturbance and contribute to the maintenance of entomopathogenic fungi, such as *Metarhizium* spp. in the soil. We design a diversified coffee system, in the Cerrado of Minas Gerais, by associating trees of *Inga edulis* (Fabaceae) and *Senna macranthera* (Fabaceae), the perennial bush *Varronia curassavica* (Cordiaceae) and non-crop plants, without pesticides. These species were selected based on the attraction and provision of plant-based food for entomophagous insects. As control, we design plots with conventional monoculture coffee with the use of pesticides. We assessed the effect of such diversification on the entomopathogenic fungi *Metarhizium*. We collected soil one month after experiment installation and after 12 and 24 months. The activity and density of *Metarhizium* spp. in coffee crop soils was assessed by using the bait survival technique with *Tenebrio molitor* (Coleoptera: Tenebrionidae), and plating soil suspensions on to solid selective media for counts of colony-forming units (CFU), respectively. We evaluated soil chemical characteristics and enzyme activity and the coffee yield from 2020. Twelve months after experiment starts, bait insects died two times faster in soil from the diversified than from conventional systems. The density of *Metarhizium* spp. was also higher in diversified system. Beta-glycosidase activity was greater in the diversified system, indicating more organic matter decomposition in this system. Furthermore, there was no difference of volume of fruit between coffee systems, but the weight of fruits was higher in diversified system in 2021. This study revealed that diversified coffee can be used as strategy in coffee crops to increases density and activity of *Metarhizium*, improves beta-glycosidase activity and coffee yield.

Keywords: Diversified coffee system; entomopathogenic fungi; ecosystem services;

[1] Chapter formatted in the norms of Agriculture, Ecosystems & Environment

1 Introduction

Soil is a reservoir for biodiversity and fungi are one of the major constituents of biodiversity (Brown et al., 2020). They are responsible for many critical ecosystem processes, such as decomposition, carbon transformation, nutrient cycling and soil structure (Singh et al., 2020). Among soil fungi, hypocrealean entomopathogens such as *Metarhizium* and *Beauveria* have been found to occur naturally in agricultural soils (Hernández-Domínguez et al., 2016; Kepler et al., 2015; Moreira et al., 2019; Sharma et al., 2018). These are important microbial control agents of insect pests, as almost all orders of insects are susceptible to them (David, 1967). Besides soil being a reservoir environment in which hypocrealean entomopathogenic fungi persist, it is also the habitat in which much of their life cycle occurs, including multiplication within hosts, saprophytic, endophytic and rhizosphere-competent growth (Elliot et al., 2000; Hesketh et al., 2010; Meyling and Eilenberg, 2007; Morgan et al., 2005; Pell et al., 2010).

Habitat structure, composition and plant diversity are important factors influencing the activity of naturally occurring hypocrealean entomopathogenic fungi (Hesketh et al., 2010). Processes such as sporulation, germination and infection of hosts require a microenvironment with high humidity (Driesche et al., 2008). Meanwhile, solar radiation is detrimental to persistence of the fungi (de Castro et al., 2013). However, agriculture intensification results in extensive areas of simplified monocultures that commonly result in proliferation of pests and pathogens, with the use of pesticides in intensively managed fields. These have largely unpredictable effects on soil biota (Brown et al., 2020), reducing soil biodiversity and causing imbalance in the ecosystem, with an oversimplification of the species (Winding et al., 2020).

Increasing plant diversification in agricultural systems can be an alternative strategy to monocultures (Barrios et al., 2018, 2012; Togni et al., 2019a, 2019b; Venzon et al., 2019). Plant diversification in agricultural systems can decrease direct solar radiation on soil, increase

microclimatic stability, promote shaded soil, conserve moisture and reduce ecosystem disturbance (Jose, 2009), which might contribute to the maintenance of the viability and virulence of soil hypocrealean entomopathogenic fungi (Moreira et al., 2019). Moreover, hypocrealean entomopathogenic fungi, especially species of *Metarhizium*, can be endophytic and associate with plant tissues such as root, stem and leaf (Ahmad et al., 2020; Bamisile et al., 2018; Batta, 2013; Behie et al., 2015, 2012; Wyrebek et al., 2011). *Metarhizium* has a cosmopolitan distribution in soils and it is a natural enemy of a wide range of arthropods (Roberts and St. Leger, 2004). Therefore, strategic diversification with plants with which *Metarhizium* is able to associate may improve its abundance and potentially increase the ecosystem services offered by these fungi to agricultural system, such as biological control (Roberts and St. Leger, 2004) and indirect protection against pest (Ahmad et al., 2020; Jaber and Araj, 2018).

A review by Vega (2018) reported several studies presenting the endophytic association of *Metarhizium* with species of trees, leguminous and grasses in several countries, including Brazil. Moreira et al. (2019) showed that bait insects exposed to soils collected in coffee agroforestry systems died faster than bait insects exposed to soils collected from coffee cultivated under full-sun. They isolated *Metarhizium* spp. from the dead bait insects in soil samples, indicating suppressive potential of pest by entomopathogenic fungi in agroforestry coffee systems and this led them to propose this methodology as way to access the ecosystem service provided by these fungi. Diversified coffee systems are common in small farms produced by family farmers in agroforestry (Ferreira, 1996). However, in most Brazilian coffee production regions, diversified coffee systems are not common, instead, are common monocultures with reduce biodiversity (Venzon, 2021). Cerrado of Minas Gerais is an important producing region of arabica coffee in Brazil, responsible for 15% of its production in the country (Conab, 2021). Patrocínio, in the Cerrado of Minas Gerais, is the municipality that

most produce coffee in Brazil, with 52,000 ha of plantation and a production of approximately 1,000,000 bags of green bean per year (Conab, 2021), but commonly in extensive areas of monoculture. Coffee was established in this region because climatic and topographical factors showed high compatibility with the crop. The flat topography contributes to intensification and technification of the coffee crop, resulting in extensive areas of full-sun monoculture agroecosystems. This scenario has potential to cause considerable biodiversity loss, microclimatic instability, soil disturbance (Jose, 2009) and decay of ecosystem services of pest control (Venzon, 2021). Therefore, these cultivated areas face considerable problems related to pests (Venzon, 2021) and can reduces *Metarhizium* spp. in soil (Moreira et al., 2019).

Considering the extensive use of monoculture coffee in the Cerrado and the largely negative effects of this on soil biota (Brown et al., 2020), our objective was to investigate whether strategic plant diversification in coffee crops can improve *Metarhizium* activity and density in the Cerrado of Minas Gerais. We assessed *Metarhizium* activity by using the bait survival technique (Moreira et al., 2019) with *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. We also assessed *Metarhizium* spp. density by plating soil suspensions on to solid selective media for counts of colony-forming units (CFU). We design a diversified system with plants that are known to attract and provide food for natural enemies of coffee pests. Thereby, we proposed a strategic diversified coffee system for *Metarhizium* conservation. The plant species were selected based on the attraction and provision of plant-based food for entomophagous insects. Our system consisted of coffee associated with trees of *Inga edulis* (Fabaceae) (Rezende et al., 2014) and *Senna macranthera* (Fabaceae), (Marazzi et al., 2013, 2006) and perennial bushes of *Varronia curassavica* (Cordiaceae) (Venzon et al., 2018). Non-crop plants were maintained between coffee rows as they can provide food resources for predators and parasitoids (Amaral et al., 2013; Venzon et al., 2019).

Besides activity and abundance of *Metarhizium* spp., we also assessed soil chemicals characteristic and microbiological activity of soils by quantification of arylsulfatase, β-glucosidase and acid phosphatase (Mendes et al., 2018). Enzyme activity is an efficient indicator of microbiological activity, as they participate as catalysts in intracellular metabolic reactions that occur in soil microbiota, involved in nutrient cycling (Ladd, 1985; Mendes et al., 2018; Miguel et al., 2020). Our objective was to evaluate whether strategic plant diversification on coffee crops improves these chemical and biological parameters that determine the quality of soils (FAO, 2020). We also estimated the coffee yield by volume of fruits per plant and weight of 100 fruits to evaluated if diversified system improves coffee yield. We hypothesize that the diversified coffee system improves (a) activity of *Metarhizium* in soil over time; (b) density of *Metarhizium* in soil over time; (c) soil enzymatic activity; (d) soil chemical characteristics over time and (e) coffee yield.

2 Material and Methods

2.1 Study area

The experiment was conducted between December 2018 and June 2021 in a non-irrigated coffee plantation at the Experimental Research Station of Agriculture and Livestock Research Enterprise of Minas Gerais (EPAMIG), in the municipality of Patrocínio, state of Minas Gerais, Brazil (18º59'48''S and 46º59'00''W, elevation ~ 950 m). The terrain is flat to gently rolling, with an average slope of 3% and the soil is classified as a Red-Yellow Latosol according to the Brazilian Soil Classification System (Kamimura et al., 2020). The region is located in the Cerrado biome, the Brazilian tropical savannah. The climate is Aw, according to the Köppen classification, with two distinct seasons, dry winter and rainy summer, with a mean annual rainfall, on the least three years, of 1,684 mm (Inmet, 2021).

The experiment was installed in December 2018 with three blocks of two plots, diversified and conventional coffee system (Figure 1A). Each plot measured 1,080 m² (30 m x 36 m) (Figure 1B and 1C). The plots were separated from each other by 200 m and the distance between blocks was at least 500 m. Blocks One and Two had *Coffea arabica,* variety "Catuaí Vermelho IAC 99", and block Three, the variety "Acaiá IAC 474 - 19". Each plot in the diversified coffee system had four *I. edulis* trees, two *S. macranthera* trees and 12 *V. curassavica* perennial bushes (Figure 1B). Non-crop plants were maintained between rows and mechanical suppression was done whenever their height exceeded 50 cm. One week before the coffee harvesting, non-crop plants were mechanically suppressed up to 10 cm, to facilitate post-harvesting treatment in the crop. No pesticides were used in the diversified coffee system., but all agronomic practices with mineral fertilization were maintained under standard coffee management of the Cerrado (Appendix 1). The plots under coffee conventional system were arranged only with coffee plants (Figure 1C). These plots were conventionally managed, implying that fertilizers and pesticides are used under standard of coffee management the Cerrado (Appendix 1).

2.2 Soil sampling

We collected soil samples in one quadrant per plot in each year to assess the activity of entomopathogenic fungi and their abundance in the two coffee systems. The arrangement of the plants associated with coffee in the diversified plots allowed the division of each plot into four quadrats, with the same number of coffee and associated plants. We collect the samples in the first quadrant (Q1) in January 2019 (one month after the experiment installation), in the fourth quadrant (Q4) in January 2020 (12 months after the experiment installation), and in the second quadrant (Q2) in January 2021 (24 months after the experiment installation) (Figure 1). In both systems, sampling was done 75 cm away from the coffee plant trunk, just beneath the

canopy, with a core soil sampler to 20 cm depth (Moreira et al., 2019). We collected 15 soil samples per plot in each year, totally 90 samples per year. Sampling was done similarly in the conventional plot following the described distances. The core soil sampler was rinsed in water, 70% ethanol and distilled water between each sample. Each soil sample was immediately transferred to individual polyethylene bag. The bags were maintained in a cold chamber at 10 ºC until transportation to the Laboratory of Entomology at EPAMIG, in Viçosa, state of Minas Gerais, Brazil.

2.3 Assessment of *Metarhizium* activity

Healthy mealworm larvae *Tenebrio molitor* (Coleoptera: Tenebrionidae) were used as bait insects. This method assesses the entomopathogenic activity of fungi present in the soil in killing the bait insects (Moreira et al., 2019). The insect larvae were obtained from a stock rearing maintained on wheat bran, chayote, carrot and potato at Laboratory of Entomology in EPAMIG, in Viçosa.

From each soil sample, a subsample of soil was transferred to a 200 ml transparent plastic pot sterilized in UV light for 15 minutes and covered with a plastic lid with four holes for ventilation. We selected five two-month-old larvae of similar size (1.3–1.5 cm) and introduced them to each plastic pot containing soil. The pots were incubated in a climate room at 25±5 ºC and 12:12 (L:D), daily shaken, inverted and left upside down to force the insects to traverse the substrate during the first 10 days. Every two days, the mortality of *T. molitor* was assessed by inspecting the pots, for 35 days. Live larvae were kept in the pots for later assessments. The pots were open next to a flame to ensure a sterile microenvironment. The samples were inspected with a sterile tweezer. Dead larvae were sterilized by immersion in 70% alcohol, 5% sodium hypochlorite, rinsed in distilled water and dried on sterile filter paper. After that, the larvae were incubated in sterile humidity chambers, made of microtubes with

moistened cotton wool soaked in distilled water, kept in a climate chamber (25±5 ºC) to promote fungal growth.

Incubated larvae were inspected daily for the presence of external fungal growth. All fungi detected were isolated on plates with PDA with 0.05 g/l chloramphenicol and incubated at 25 ºC. Under a microscope (40x objective), we prepared slides of the isolates' reproductive structures for morphological identification. After, we calculated the proportion of bait insect infected by *Metarhizium* per soil sample and the survival of bait insect.

2.4 Assessment of *Metarhizium* density

To evaluate the densities of *Metarhizium* spp., fungal isolation from sampled soil was done for counts of colony-forming units (CFU). We used a selective media that favors the growth of Hypocrealean fungi and we evaluated the total CFU, which is the number of CFU of all fungi grow up in the plates. Besides, we evaluated only the number of CFU of *Metarhizium* spp. For each soil sample, 5 g of soil was diluted in 45 ml of sterile distilled water solution of Tween 0.01% in a Falcon® tube. Tubes were rotated for one hour in a rotary shaker at 150 rpm (Lacey, 2012). The suspensions were vortexed for 15 s and plated on to selective culture media. We used a selective medium with 10 g peptone, 20 g dextrose, 15 g agar per litter of distilled water. After sterilizing the medium, we added 0.05 g/l of cycloheximide and tetracycline, 0.6 g/l streptomycin and 0.175 g/l CTAB (cetyltrimethylammonium bromide) (Kepler et al., 2015). One hundred microliters from each suspension were plated in three Petri dishes (9 cm diameter) and spread with a sterile Drigalski spatula. Plates were kept in the dark at 25 °C for 14 days until CFU counts could be done. The densities of CFU per gram of soil were calculated by multiplying the number of CFU (mean of three plates) by 100, as 100 µl of the soil suspension corresponds to 0.01 g soil. From the 2020 field collection, each *Metarhizium* colony detected was morphologically identified and counted.

2.5 Enzymatic analyses

We collected 20 soil samples to 10 cm depth per plot in January 2021. The sampling was done randomly throughout the whole plot, between rows and beneath the canopy. The 20 samples were mixed for a composite sample per plot, totalizing six samples, one per plot. We send the samples to Laboratory of Genomic Analysis and Biotechnology where the quantification of Arylsulfatase, β-glycosidase and Acid phosphatase were done.

2.6 Soil chemical and texture analysis

We collected five soil samples (75 cm away from the coffee plant trunk, just beneath the canopy, with a core soil sampler to 20 cm depth) per plot in January 2019, January 2020 and January 2021. The five samples were mixed for a composite sample per plot, totaling six samples each year. Soil texture, chemical and organic matter were analyzed at the Soil, Vegetable Tissue and Fertilizer Laboratory of Federal University of Viçosa (Table 1). Soil texture analysis was done one month after the experiment was installed, just to know the characteristics of the experimental plots, because clay, silt and sand content do not change with different managements (Van Lier, 2010).

2.7 Coffee yield

The harvest was carried out in June 2020 and May 2021. We did not use de yield of 2019, because conventional agronomic practices were maintained in the experimental area until December 2018, when we installed the experiment. In this period occurred the bloom and coffee fruit formation., therefore, the coffee production in 2019 was influenced by 2018 management. We collected all fruits from 24 coffee plants per plot in each year to measure the volume of coffee fruits of each plant with a graduated bucket. We also weighed 100 fruits from each plant to estimate the weight of the fruits (g/100 fruits).

2.8 Statistical analyses

Analyses were performed using the R statistical software (R Core Team, 2017). We used system (i.e., diversified and conventional coffee systems) and year (i.e., 2019, 2020 and 2021) as explanatory variables to investigate how strategic diversification affects abundance and activity of *Metarhizium* spp. and enzymatic activity in coffee crops. Survival analyses was carried out to test time to death of *T. molitor* larvae bait in soil samples from diversified and conventional coffee systems. We included solely the dead insects killed by *Metarhizium* spp. We used the R software package "frailtypack" (Rondeu et al., 2019) to added a penalization in the models, with gamma distributions. The penalization was added to fit a joint frailty model of the five insects in same soil sample. Analyses were ANOVA with χ^2 tests. To examine the proportion of *Metarhizium* spp. found in bait insects, we used proportion of larvae infected per soil sample and used a generalized linear model (GLM) with binomial distribution, appropriate for proportion data,and analyzed by ANOVA with χ^2 tests (Crawley, 2015). To test the numbers of total CFU and *Metarhizium* spp. CFU, we used a GLM with Poisson distributions and analyzed by ANOVA with χ^2 tests. For enzymatic activity, we used Analysis of Deviance. Soil chemicals were classified according to Ribeiro et al. (1999) and texture characteristic were classified by soil textural triangle (Ribeiro et al., 1999). Because the soil chemical and texture characteristics classification of all soil samples was in the same pattern, we did not perform statistical analysis to compared the coffee systems and years. We analyzed volume of fruits per plant and weight of fruits by Analysis of Deviance.

3 Results

3.1 Survival of bait insects

The survival of bait insects was influenced by coffee systems (χ^2 =15.91, p<0.001) and years (χ^2 =109.22, p<0.001). In 2019, one month after the experiment starts, the survival was

similar between the coffee systems (t=2.02, p=0.32, Figure 2). However, 12 months after experiment establishment, bait insects died two times faster due to *Metarhizium* spp. in soil from the diversified, than in soil from the conventional coffee systems. After 35 days in 2020, 47.11 ±3.33% of bait insects were dead by *Metarhizium* spp. in the soil from diversified system, whereas 28.88±3.02% were dead in the conventional 2020 (t=3.96, p=0.0011, Figure 2). In 2021, 36.88±3.19 of bait insects were dead by *Metarhizium* spp. in the soil from diversified system, whereas 20.44±2.67% were dead in the conventional (t=3.62, p=0.0041, Figure 2). Over time, the survival of bait insect decreased in 2020 compared to 2019 in both coffee systems (conventional: t=4.14, p<0.001; diversified: t=8.19, p<0.001). However, the survival did not differ between 2020 and 2021 in both systems (conventional: t=2.40, p=0.15; diversified: t=2.80, p=0.05). In 2021 the survival decreased in the diversified (t=6.45, p<0.001) and did not differ in the conventional coffee system (t=1.88, p=0.414), compared to 2019.

3.2 Proportion of *Metarhizium* spp.

The proportion of bait insects infected by *Metarhizium s*pp. was influenced by the coffee systems (χ^2 =18.49, p=0.001) and years (χ^2 =96.28, p<0.001). It was similar one month after the experiment starts in both coffee systems (conventional *vs* diversified coffee system: 0.14±0.03 *vs* 0.088±0.02; z=1.35, p=0.75, Figure 3). However, one year (conventional *vs* diversified coffee system: 0.28±0.04 *vs* 0.47±0.05; z=3.171, p=0.01) and two years later (conventional *vs* diversified coffee system: 0.19±0.03 *vs* 0.33±0.03; z=2.92, p<0.01) after, the proportion was higher in the diversified coffee system (Figure 3). Over time, the proportion was similar in the conventional coffee system (2019 *vs* 2020: z=2.66, p=0.082; 2020 *vs* 2021: z=1.98, p=0.35; 2019 *vs* 2021: z=0.73, p=0.97), but it increased in 2020 (z=6.20, p<0.001) and 2021 (z=4.42, p<0.001), compared to 2019, in the diversified system. There was no difference in the diversified coffee system between 2020 and 2021 (z=2.40, p=0.15).

3.3 Fungi density

Metarhizium spp. density, measured as CFU of *Metarhizium* spp. per gram of soil, was influenced by systems (χ^2=48132, p<0.001) and years (χ^2=479057, p<0.001, Figure 4A). CFU of *Metarhizium* spp. was significantly higher in diversified coffee system in 2020 (conventional vs diversified coffee system: 3385.81±755.83 vs 6357.77±911.73 CFU/g soil; z=3.23, p=0.0066, Figure 4A) and 2021 (conventional vs diversified coffee system: 57.77±24.76 vs 351.11± 92.54 CFU/g soil; z= 2.82, p=0.0059, Figure 4A), than conventional coffee system. Over time, *Metarhizium* spp. CFU decreases in 2021 compared to 2020 in both coffee systems (conventional: z=3.35, p=0.004, diversified: z=5.77, p<0.001).

Total fungi density, measured as CFU total per gram of soil, was not influenced by the coffee systems (χ^2=2.10, p=0.14), but it was influenced by the years (χ^2=432.11, p<0.001, Figure 4B). The total CFU increases in 2020 compared to 2019 in both conventional (2019 *vs* 2020: 1455.55±341.20 *vs* 15591.11±1866.75 CFU/g soil; z=14.60, p<0.001) and diversified coffee system (2019 *vs* 2020: 1194.81±251.70 vs 12954.07±1569.65 CFU/g soil; z= 14.60, p<0.001). However, the total CFU decreases in 2021 compared to 2020 in both systems (conventional *vs* diversified coffee system: 331.85±37.84 vs 613.33±99.42 CFU/ g soil; z=21.085 p<0.001). Also, the total CFU decreases in 2021 compared to 2019 in both coffee systems (z=6.48, p<0.001).

3.4 Enzymatic quantification

Beta-glucosidase activity was greater in the soil of diversified than in the conventional coffee system (conventional *vs* diversified coffee system= 684.10±23.32 *vs* 794.79±18.01 μg PNG. g^{-1} soil. h^{-1}; F=23.02, p<0.01, Figure 5). The acid phosphatase (conventional *vs* diversified coffee system: 735.74±4.64 vs 731.18±8.32 01 μg PNP. g^{-1} soil. h^{-1}; F=0.215,

p=0.650 and arylsulfatase (conventional *vs* diversified coffee system: 413.86±14.03 *vs* 424.99±19.74 µg PNS. g^{-1} soil. h^{-1}; F=0.222, p=0.644) were the same in both coffee systems.

3.5 Soil chemical and texture characteristics

All the samples had similar soil chemical and texture characteristics in the three years, with lower pH (4.44-5.78), medium organic matter (3.59%-4.96%), and high clay contents (>67%) in the three years (Table 1).

3.6 Coffee yield

The volume of coffee fruits per plant did not differ between diversified and conventional systems in both years (2020: F=1.343, p=0.249; 2021: F=3.809, p=0.0530, Figure 6A). In 2020, the volume was 7.98±0.58 l/plant in the conventional and 7.12±0.45 l/plant in the diversified coffee system. In 2021, the volume was 11.49±0.99 l/plant in the conventional and 9.09±0.79 l/plant in the diversified coffee system. In 2020, the weight of 100 fruits was higher in conventional system (conventional *vs* diversified: 13.04±2.45 *vs* 107.19±1.92 g/100 fruits; F=5.608, p=0.0193, Figure 6B). However, the weight of 100 fruits was higher in diversified system in 2021 (conventional *vs* diversified: 118.86±2.37 *vs* 125.15±2.08 g/100 fruits; F=7.081, p=0.0087, Figure 6B).

4 Discussion

Our findings show that strategic diversification of coffee crops with *I. edulis*, *S. macranthera*, *V. curassavica* and non-crop plants improve the maintenance and virulence of *Metarhizium* spp. in coffee crop soil. Possibly, the presence of these plants decreased solar radiation incidence on soil and, consequently, increased soil humidity in the diversified coffee

systems, two important environmental factors that affect the action of entomopathogens in the field (Braga et al., 2001; Driesche et al., 2008; Lovett and St. Leger, 2015). Solar radiation is detrimental to the fungi (de Castro et al., 2013). Besides, the germination, infection, and sporulation of fungi requires a microenvironment with high humidity (Driesche et al., 2008).

Metarhizium spp. are more common entomopathogenic fungi in agricultural soils (Botelho et al., 2019; Moreira et al., 2019) and are natural enemies of a wide range of arthropods (Brunner-Mendoza et al., 2019; David, 1967; Jaramillo et al., 2015; Meyling and Eilenberg, 2007; Wraight et al., 2018). However, sustainable agriculture systems, such as diversified and organic systems increase the occurrence of *Metarhizium* (Clifton et al., 2015). Moreira et al. (2019) found that *Metarhizium* spp. Activity is greater in soil samples from organic agroforestry coffee systems than full-sun coffee systems in Araponga, Zona da Mata Mineira region, within the Atlantic Coastal Rainforest biome. The region is characterized by agroforestry with a tropical highland climate with mean temperature and precipitation of 18°C and 1,500 mm (Inmet, 2021). We found similar results when coffee was diversified, but in a different biome, Cerrado, with high-altitude humid subtropical, with mean temperature of 21.4°C and 1,684 mm (Inmet, 2021). Therefore, designing strategic diversified coffee system at Cerrado can increases microclimatic stability within the crop, thus improving soil microbiota.

The genus *Metarhizium* tolerates a periodic absence of arthropods due to colonization in different plants (Behie et al., 2012; Behie and Bidochka, 2014; Fisher et al., 2011; Keyser et al., 2015; Krell et al., 2018; Morgan et al., 2005), besides the conidia ability to resist the harmful influence of abiotic factors and/or biodegradation in the soil (Vänninen, 1996). When endophytically colonize plants, *Metarhizium* can promote plant growth and protection against pests (Behie et al., 2012; Behie and Bidochka, 2014; Jaber and Araj, 2018). It is also able to improve plant nutrient acquisition when associated with plant roots (Behie et al., 2012; Behie and Bidochka, 2014). In 2020, the weight of fruits was higher in conventional system, but one

year later the weight was higher in diversified system. These results suggest that over time, the strategic diversification can improve coffee yield. Rezende et al. (2021) found higher coffee production on coffee plants closer to the *Inga* trees, which is one of the plants that we associated with coffee plants. It is possible that the higher density of *Metarhizium* spp. in the diversified system contributed with plant nutrient acquisition, consequently, increasing the coffee yield over time. Accordingly, the adoption of coffee systems which improve the maintenance of *Metarhizium* spp. can contribute to natural control of soil pests, promotes plant growth and protection against pests.

Metarhizium spp. density, survival of bait insects and proportion of bait insect infected by *Metarhizium* spp. oscillated between the years in both diversified and conventional systems. This can be partially explained by the difference in the rainfall regime between years. Three months before the soil was collected, in January 2019, the precipitation was 693 mm, whereas it was 893.08 mm in the same period in 2020, and 441.26 mm in 2021 (Inmet, 2021). The fungi require a microenvironment with high humidity (Driesche et al., 2008), therefore, the higher precipitation level in 2020 can explained more fungi abundance and activity than 2021. Though these factors oscillated between years in both systems, from 2020 the *Metarhizium* spp. abundance and proportion of bait insect infected by *Metarhizium* spp. was higher in the diversified coffee system. Besides, the survival of bait insect was lower in the diversified than conventional coffee system, showing that the strategic diversification benefits the maintenance of them both in the rainy periods as in drought period.

Besides *Metarhizium* spp. activity and density, in the diversified coffee system we found more beta-glucosidase enzyme activity than in the conventional coffee system. Soil enzyme activity is closely related to soil nutrient cycling and respond rapidly to soil changes (Cui et al., 2019), and beta-glucosidase is involved to organic matter decomposition and carbon cycle (Bandick and Dick, 1999). A meta-analysis about soil quality indicators carried out by Gil-

Sotres et al. (2005) showed that this enzyme is considered as indicator and discriminator for differentiating soil quality. Therefore, the higher beta-glucosidase enzyme activity in the diversified coffee system indicate that the strategic plant diversification improves the soil quality, benefits organic matter decomposition and, consequently, soil nutrient availability (Pavinato and Rosolem, 2008). Possibly, higher activity of beta-glycosidase together with the higher density of *Metarhizium* in soil have contributed to increase in coffee yield.

Soil texture and chemical factors influence the occurrence of entomopathogenic fungi (Clifton et al., 2015; Quesada-moraga et al., 2007). The proportion of silt positively correlates with *Metarhizium* spp. abundance (Clifton et al., 2015). Also, *Metarhizium* spp. have positive correlation with higher levels of organic matter (Quesada-moraga et al., 2007). In our study, the soil properties did not show variation between conventional and diversified coffee systems. All the samples had lower pH, medium organic matter and high clay contents for coffee crop (Ribeiro et al., 1999). Therefore, there was not specific relation to soil properties in the occurrence of *Metarhizium* spp. Besides, these results indicating that our strategic diversification did not interfere in the soil chemical factors until now. Chemical factors, such as soil organic matter, change very slowly, therefore, many years may be required to measure changes resulting from restauration (Dick, 1992). Possibly, there was not sufficient time to strategic diversification improves soil chemical factors. However, enzymatic analyses which respond rapidly to soil changes (Cui et al., 2019) indicating that diversified coffee system can improve soil chemical factors over time.

Our study showed high density and activity of *Metarhizium* spp., beta-glucosidase activity and coffee yield in diversified coffee system. Our strategic diversification was designed with plants that are known to mediate important ecosystem services. *Inga edulis* is a Leguminosae tree that fix nitrogen in soil (Tully et al., 2012). Besides, this tree has extrafloral nectaries which improve the maintenance of natural enemies of coffee pests (Rezende et al.,

2014, 2021). *Senna macranthera* is found associated with coffee in agroforestry systems (Souza et al., 2010) and it also Leguminosae tree that fix nitrogen in soil and has extrafloral nectaries (Marazzi et al., 2013). *Varronia currasavica* blooms all year round, providing a constant source of pollen and nectar which serve as food to natural enemies (Venzon et al., 2018). The maintenance of non-crop plants also provides alternative food and refuge for natural enemies (Amaral et al., 2013; Togni et al., 2019b; Venzon et al., 2019). Thus, together with other ecosystem services, such as pollination and biological control by entomophagous (Amaral et al., 2016; Fiedler et al., 2008; Rezende et al., 2014; Togni et al., 2019b, 2019a; Venzon et al., 2019), plant diversification can conserve *Metarhizium* spp. on soil and increases coffee yield

5 Acknowledgments

The present study was supported by ‘Conselho Nacional de Desenvolvimento Científico e Tecnológico’ (CNPq), ‘Fundação de Amparo a Pesquisa de Minas Gerais’ (FAPEMIG), 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil' (CAPES) - Finance Code 001 and Consórcio Brasileiro de Pesquisa e Desenvolvimento do Café (CBP&D-Café). We are grateful Jaime Silva, Diego Vilela, Adalberto (Nenzinho), Ronaldo Gaspar, Hélio Bernardes and the coffee harvest workers of EPAMIG - Patrocínio for all their support in carrying out the field experiments. We thank Marcela Caixeta, Keminy Bautz, Elenir Queiroz, Walysson Mendes, Eduardo Costantin and Verônica Fialho, from the laboratory of Insect-Microorganism Interactions, UFV, for help with laboratory protocols. We also thank Jéssica Martins for her support in preparing the sketch of the experimental design.

6 References

Ahmad, I., Jiménez-Gasco, M.M., Luthe, D.S., Shakeel, S.N., Barbercheck, M.E., 2020. Endophytic *Metarhizium robertsii* promotes maize growth, suppresses insect growth, and

alters plant defense gene expression. Biol. Control 144, 104167. https://doi.org/10.1016/j.biocontrol.2019.104167

Amaral, D.S.S.L., Venzon, M., Duarte, M.V.A., Sousa, F.F., Pallini, A., Harwood, J.D., 2013. Non-crop vegetation associated with chili pepper agroecosystems promote the abundance and survival of aphid predators. Biol. Control 64, 338–346. https://doi.org/10.1016/j.biocontrol.2012.12.006

Amaral, D.S.S.L., Venzon, M., Helder, H., Sujii, E.R., Schmidt, J.M., Harwood, J.D., 2016. Non-crop plant communities conserve spider populations in chili pepper agroecosystems. Biol. Control 103, 69–77. https://doi.org/10.1016/j.biocontrol.2016.07.007

Bamisile, B.S., Dash, C.K., Akutse, K.S., Keppanan, R., Wang, L., 2018. Fungal endophytes: Beyond herbivore management. Front. Microbiol. 9, 544. https://doi.org/10.3389/fmicb.2018.00544

Bandick, A.K., Dick, R.P., 1999. Field management effects on soil enzyme activities. Soil Biol. Biochem. 31, 1471–1479. https://doi.org/10.1016/S0038-0717(99)00051-6

Barrios, E., Sileshi, G.W., Shepherd, K., Sinclair, F., 2012. Agroforestry and soil health: Linking trees, soil biota, and ecosystem services, in: Wall, D.H., Bardgett, R.D., Behan-Pelletier, V., Herrick, J.E., Jones, T.H., Ritz, K., Six, J., Strong, D.R., Putten, W. van der (Eds.), Soil Ecology and Ecosystem Services. Oxford University Press, Oxford UK, pp. 315–330.

Barrios, E., Valencia, V., Jonsson, M., Brauman, A., Hairiah, K., Mortimer, P.E., Okubo, S., 2018. Contribution of trees to the conservation of biodiversity and ecosystem services in agricultural landscapes. Int. J. Biodivers. Sci. Ecosyst. Serv. Manag. 14, 1–16. https://doi.org/10.1080/21513732.2017.1399167

Batta, Y.A., 2013. Efficacy of endophytic and applied *Metarhizium anisopliae* (Metch.) Sorokin (Ascomycota : Hypocreales) against larvae of *Plutella xylostella* L.

(Yponomeutidae : Lepidoptera) infesting *Brassica napus* plants. Crop Prot. 44, 128–134. https://doi.org/10.1016/j.cropro.2012.11.001

Behie, S.W., Bidochka, M.J., 2014. Nutrient transfer in plant–fungal symbioses. Trends Plant Sci. 19, 734–740. https://doi.org/10.1016/j.tplants.2014.06.007

Behie, S.W., Jones, S.J., Bidochka, M.J., 2015. Plant tissue localization of the endophytic insect pathogenic fungi *Metarhizium* and *Beauveria*. Fungal Ecol. 13, 112–119. https://doi.org/10.1016/j.funeco.2014.08.001

Behie, S.W., Zelisko, P.M., Bidochka, M.J., 2012. Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. Science. 336, 1576–1577. https://doi.org/10.1126/science.1222289

Botelho, A.B.R.Z., Alves-Pereira, A., Prado, R.C., Zucchi, M.I., Delalibera Júnior, I., 2019. *Metarhizium* species in soil from Brazilian biomes: a study of diversity, distribution, and association with natural and agricultural environments. Fungal Ecol. 41, 289–300. https://doi.org/10.1016/j.funeco.2019.07.004

Braga, G.U.L., Flint, S.D., Miller, C.D., Anderson, A.J., Roberts, D.W., 2001. Both solar UVA and UVB radiation impair conidial culturability and delay germination in the entomopathogenic fungus *Metarhizium anisopliae*. Photochem. Photobiol. 74, 734–739. https://doi.org/10.1562/0031-8655(2001)074<0734:bsuaur>2.0.co;2

Brown, G., Cooper, M., Kobayashi, M., 2020. Threats to soil biodiversity – global and regional trends, in: FAO, ITPS, GSBI, CBD, EC (Eds.), State of Knowledge of Soil Biodiversity: Status, Challenges and Potentialities. FAO, Roma, pp. 191–262.

Brunner-Mendoza, C., Reyes-Montes, M. del R., Moonjely, S., Bidochka, M.J., Toriello, C., 2019. A review on the genus *Metarhizium* as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico. Biocontrol Sci. Technol. 29, 83–102. https://doi.org/10.1080/09583157.2018.1531111

Clifton, E.H., Jaronski, S.T., Hodgson, E.W., Gassmann, A.J., 2015. Abundance of soil-borne entomopathogenic fungi in organic and conventional fields in the Midwestern USA with an emphasis on the effect of herbicides and fungicides on fungal persistence. PLoS One 10, e0133613. https://doi.org/10.1371/journal.pone.0133613

Conab, 2021. Acompanhamento da Safra Brasileira: Café. Companhia Nacional de Abastecimento URL https://www.conab.gov.br/index.php/info-agro/safras/cafe (accessed 9.20.21).

Crawley, M.J., 2015. Statistics: An introduction using R, 2nd ed. John Wiley & Sons, Ltd, United kingdom.

Cui, Y., Fang, L., Guo, X., Wang, X., Wang, Y., Zhang, Y., Zhang, X., 2019. Responses of soil bacterial communities, enzyme activities, and nutrients to agricultural-to-natural ecosystem conversion in the Loess Plateau, China. J. Soils Sediments 19, 1427–1440. https://doi.org/10.1007/s11368-018-2110-4

David, W.A.L., 1967. The physiology of the insect integument in relation to the invasion of pathogens, in: Beament, J.W.L.; Treherne, J.E. (Ed.), Insects and Physiology. Edinburgh, &c., Oliver & Boyd., pp. 17–35.

de Castro, T.R., Wekesa, V.W., Moral, R.D.A., Demétrio, C.G.B., Delalibera, I., Klingen, I., 2013. The effects of photoperiod and light intensity on the sporulation of brazilian and norwegian isolates of *Neozygites floridana*. J. Invertebr. Pathol. 114, 230–233. https://doi.org/10.1016/j.jip.2013.08.004

Dick, R.P., 1992. A review: long-term effects of agricultural systems on soil biochemical and microbial parameters. Agric. Ecosyst. Environ. 40, 25–36. https://doi.org/10.1016/0167-8809(92)90081-L

Driesche, R. Van, Hoddle, M., Center, T., 2008. Control of pests and weeds by natural enemies: An introduction to biological control, 1st ed. Blackwell publishing Ltd, Oxford UK.

https://doi.org/10.1653/024.092.0237

Elliot, S.L., Sabelis, M.W., Janssen, A., Van der Geest, L.P.S., Beerling, E.A.M., Fransen, J., 2000. Can plants use entomopathogens as bodyguards? Ecol. Lett. 3, 228–235. https://doi.org/10.1046/j.1461-0248.2000.00137.x

FAO, 2020. State of knowledge of soil biodiversity: Status, challenges and potentialities, 1st ed. FAO, Roma.

Ferreira, E.A., 1996. Desenvolvimento da Agricultura Familiar: a experiência do CTA-ZM, in: Alvares, V.H., Fontes, L.E.F., Fontes, M.P.F. (Eds.), O Solo Nos Grandes Domínios Morfoclimáticos Do Brasil e o Desenvolvimento Sustentado. Sociedade Brasileira de Ciência do Solo, Viçosa, pp. 233–250.

Fiedler, A.K., Landis, D.A., Wratten, S.D., 2008. Maximizing ecosystem services from conservation biological control: The role of habitat management. Biol. Control 45, 254–271. https://doi.org/10.1016/j.biocontrol.2007.12.009

Fisher, J.J., Rehner, S.A., Bruck, D.J., 2011. Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. J. Invertebr. Pathol. 106, 289–295. https://doi.org/10.1016/j.jip.2010.11.001

Gil-Sotres, F., Trasar-Cepeda, C., Leirós, M.C., Seoane, S., 2005. Different approaches to evaluating soil quality using biochemical properties. Soil Biol. Biochem. 37, 877–887. https://doi.org/10.1016/j.soilbio.2004.10.003

Hernández-Domínguez, C., Cerroblanco-Baxcajay, M. de L., Alvarado-Aragón, L.U., Hernández-López, G., Guzmán-Franco, A.W., 2016. Comparison of the relative efficacy of an insect baiting method and selective media for diversity studies of *Metarhizium* species in the soil. Biocontrol Sci. Technol. 26, 707–717. https://doi.org/10.1080/09583157.2016.1152458

Hesketh, H., Roy, H.E., Eilenberg, J., Pell, J.K., Hails, R.S., 2010. Challenges in modelling

complexity of fungal entomopathogens in semi-natural populations of insects. BioControl 55, 55–73. https://doi.org/10.1007/s10526-009-9249-2

Inmet, 2021. Instituto Nacional de Metereologia. URL https://clima.inmet.gov.br/prec (accessed 7.8.21).

Jaber, L.R., Araj, S.E., 2018. Interactions among endophytic fungal entomopathogens (Ascomycota: Hypocreales), the green peach aphid *Myzus persicae* Sulzer (Homoptera: Aphididae), and the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae). Biol. Control 116, 53–61. https://doi.org/10.1016/j.biocontrol.2017.04.005

Jaramillo, J.L., Montoya, E.C., Benavides, P., Góngora B, C.E., 2015. *Beauveria bassiana* y *Metarhizium anisopliae* para el control de broca del café en frutos del suelo. Rev. Colomb. Entomol. 41, 95–104.

Jose, S., 2009. Agroforestry for ecosystem services and environmental benefits: An overview. Agrofor. Syst. 76, 1–10. https://doi.org/10.1007/s10457-009-9229-7

Kamimura, K.M., Dias Júnior, M. de S., de Oliveira, M.S., Dos Santos, G.R., Guimarães, P.T.G., Ajayi, A.E., 2020. Spatial variability of precompression stress and volumetric water content of a red-yellow latosol (Oxisol). Biosci. J. 36, 142–151. https://doi.org/10.14393/BJ-v36n1a2020-42121

Kepler, R.M., Ugine, T.A., Maul, J.E., Cavigelli, M.A., Rehner, S.A., 2015. Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. Environ. Microbiol. 17, 2791–2804. https://doi.org/10.1111/1462-2920.12778

Keyser, C.A., De Fine Licht, H.H., Steinwender, B.M., Meyling, N. V., 2015. Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. BMC Microbiol. 15, 249. https://doi.org/10.1186/s12866-015-0589-z

Krell, V., Jakobs-Schoenwandt, D., Vidal, S., Patel, A. V, 2018. Cellulase enhances

endophytism of encapsulated *Metarhizium brunneum* in potato plants. Fungal Biol. 122, 373–378. https://doi.org/10.1016/j.funbio.2018.03.002

Lacey, L.A., 2012. Manual of techniques in invertebrate pathology, 2nd ed. Elsevier Ltd., Yakima, Washington, EUA.

Ladd, J.N., 1985. Soil enzymes, in: D, V., R.E, M. (Eds.), Origin and Range of Enzymes in Soil. Developments in Plant and Soil Sciences. Springer, Dordrecht, pp. 175–221.

Lovett, B., St. Leger, R.J., 2015. Stress is the rule rather than the exception for *Metarhizium*. Curr. Genet. 61, 253–261. https://doi.org/10.1007/s00294-014-0447-9

Marazzi, B., Conti, E., Sanderson, M.J., Mcmahon, M.M., Bronstein, J.L., 2013. Diversity and evolution of a trait mediating ant-plant interactions: Insights from extrafloral nectaries in *Senna* (Leguminosae). Ann. Bot. 111, 1263–1275. https://doi.org/10.1093/aob/mcs226

Marazzi, B., Fortunato, R.H., Endress, P.K., Spichiger, R., 2006. *Senna* (Cassiinae, Leguminosae) in Paraguay: Synopsis, occurrence, ecological role and ethnobotany. Candollea 61, 315–329.

Mendes, I.C., Sousa, D.M.G. de, Reis Junior, F.B. dos, Lopes, A.A. de C., 2018. Bioanálise de solo: como acessar e interpretar a saúde do solo. Circ. Técnica - Embrapa Cerrados 38, 23.

Meyling, N. V, Eilenberg, J., 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems : Potential for conservation biological control. Biol. Control 43, 145–155. https://doi.org/https://doi.org/10.1016/j.biocontrol.2007.07.007

Miguel, D.L., da Silva, E.M.R., da Silva, C.F., Pereira, M.G., Leite, L.F.C., 2020. Soil microbiological properties and enzyme activity in agroforestry systems compared with monoculture, natural regeneration, and native caatinga. Biosci. J. 36, 1–16. https://doi.org/10.14393/BJ-v36n1a2020-42704

Moreira, C.C., Celestino, D., Guerra, T., Cardoso, I.M., Elliot, S.L., 2019. Agroforestry coffee

soils increase the insect-suppressive potential offered by entomopathogenic fungi over full-sun soils : A case proposing a “bait survival technique.” Ecol. Evol. 9, 10777–10787. https://doi.org/10.1002/ece3.5598

Morgan, J.A.W., Bending, G.D., White, P.J., 2005. Biological costs and benefits to plant-microbe interactions in the rhizosphere. J. Exp. Bot. 56, 1729–1739. https://doi.org/10.1093/jxb/eri205

Pavinato, P.S., Rosolem, C.A., 2008. Disponibilidade de nutrientes no solo: decomposição e liberação de compostos orgânicos de resíduos vegetais. Rev. Bras. Ciênc. Solo 32, 911-920. https://doi.org/10.1590/S0100-06832008000300001

Pell, J.K., Hannam, J.J., Steinkraus, D.C., 2010. Conservation biological control using fungal entomopathogens. BioControl 55, 187–198. https://doi.org/10.1007/s10526-009-9245-6

Quesada-moraga, E., Navas-corte, J.A., Matanhao, E.A.A., Ortiz-Urquiza, A., Santiago-Álavrez, C., 2007. Factors affecting the occurrence and distribution of entomopathogenic fungi in natural and cultivated soils. Mycol. Res. 111, 947–966. https://doi.org/10.1016/j.mycres.2007.06.006

Rezende, M.Q., Venzon, M., Santos, P.S., Cardoso, I.M., Janssen, A., 2021. Extrafloral nectary-bearing leguminous trees enhance pest control and increase fruit weight in associated coffee plants. Agric Ecosyst Environ 319, 107538. https://doi.org/10.1016/j.agee.2021.107538

Rezende, M.Q., Venzon, M., Perez, A.L., Cardoso, I.M., Janssen, A., 2014. Extrafloral nectaries of associated trees can enhance natural pest control. Agric. Ecosyst. Environ. 188, 198–203. https://doi.org/10.1016/j.agee.2014.02.024

Ribeiro, A.C., Guimarães, P.T.G., Alvarez V, V.H., 1999. Recomendações para o uso de corretivos e fertilizantes em Minas Gerais - 5ª Aproximação, Recomendações para o uso de corretivos e fertilizantes em Minas Gerais - 5° Aproximação. Comissão de Fertilidade

do Solo do Estado de Minas Gerais - CFSEMG, Viçosa.

Roberts, D.W., St. Leger, R.J., 2004. *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. Adv. Appl. Microbiol. 54, 1–70. https://doi.org/10.1016/S0065-2164(04)54001-7

Rondeau, V., Gonzalez, J.R., Yassin Mazroui, A. Mauguen, A.D., Laurent, A., Lopez, M., Rondeau, M. V., 2019. Package 'frailtypack'.

Sharma, L., Oliveira, I., Torres, L., Marques, G., 2018. Entomopathogenic fungi in Portuguese vineyards soils: suggesting a 'Galleria-Tenebrio-bait method' as bait- insects *Galleria* and *Tenebrio* significantly underestimate the respective recoveries of *Metarhizium* (*robertsii*) and *Beauveria* (*bassiana*). MycoKeys 38, 1–23. https://doi.org/10.3897/mycokeys.38.26970

Singh, B.K., Nico Eisenhauer, Zöe Lindo, 2020. Global diversity and distribution of soil biodiversity, in: FAO, ITPS, GSBI, CBD, EC (Eds.), State of Knowledge of Soil Biodiversity: Status, Challenges and Potentialities. FAO, Roma, pp. 7–114.

Souza, H.N., Cardoso, I.M., Fernandes, J.M., Garcia, F.C.P., Bonfim, V.R., Santos, A.C., Carvalho, A.F., Mendonça, E.S., 2010. Selection of native trees for intercropping with coffee in the Atlantic Rainforest biome. Agrofor. Syst. 80, 1–16. https://doi.org/10.1007 / s10457-010-9340-9

Togni, P.H.B., Venzon, M., Souza, L.M., Santos, J.P.C.R., Sujii, E.R., 2019a. Biodiversity provides whitefly biological control based on farm management. J. Pest Sci. (2004). 92, 393–403. https://doi.org/10.1007/s10340-018-1021-x

Togni, P.H.B., Venzon, M., Souza, L.M., Sousa, A.A.T.C., Harterreiten-souza, É.S., Pires, C.S.S., Sujii, E.R., 2019b. Dynamics of predatory and herbivorous insects at the farm scale: the role of cropped and noncropped habitats. Agric. For. Entomol. 21, 351–362. https://doi.org/10.1111/afe.12337

Tully, K.L., Lawrence, D., Scanlon, T.M., 2012. More trees less loss: nitrogen leaching losses decrease with increasing biomass in coffee agroforests. Agric. Ecosyst. Environ. 161, 137–144. https://doi.org/10.1016/j.agee.2012.08.002

Van Lier, Q.J. Física do Solo. Sociedade Brasileira de Ciência do Solo, Viçosa, 298p.

Vänninen, I., 1996. Distribution and occurrence of four entomopathogenic fungi in Finland: Effect of geographical location, habitat type and soil type. Mycol. Res. 100, 93–101. https://doi.org/10.1016/S0953-7562(96)80106-7

Vega, F.E., 2018. The use of fungal entomopathogens as endophytes in biological control: a review. Mycologia 110, 4–30. https://doi.org/10.1080/00275514.2017.1418578

Venzon, M., 2021. Agro-ecological management of coffee pests in Brazil. Front. Sustain. Food Syst. 5, 721117. https://doi.org/10.3389/fsufs.2021.721117

Venzon, M., Togni, P.H.B., Chiguachi, J.A.M., Pantoja, G.M., Brito, E.A.S., Sujii, E.R., 2019. Agrobiodiversidade como estratégia de manejo de pragas. Inf. Agropecuário 40, 21–29.

Venzon, M., Togni, P.H.B., Amaral, D.S.S.L., Rezende, M.Q., Batista, M.C., Chiguachi, J.A.M., Martins, E.F., Perez, A.L., 2018. Mobilisation des mécanismes de régulation naturelle des ravageurs via des plantes à multiples services écosystémiques. Innov. Agron. 64, 83–95.

Winding, A., Bach, E., Mele, P., 2020. Contributions of soil biodiversity to ecosystem functions and services, in: FAO, ITPS, GSBI, CBD, EC (Eds.), State of Knowledge of Soil Biodiversity: Status, Challenges and Potentialities. FAO, Roma, pp. 115–190.

Wraight, S.P., Galaini-Wraight, S., Howes, R.L., Castrillo, L.A., Carrunthers, R.I., Smith, R.H., Matsumoto, T.K., Keith, L.M., 2018. Prevalence of naturally-occurring strains of *Beauveria bassiana* in populations of coffee berry borer *Hypothenemus hampei* on Hawai'i Island, with observations on coffee plant-*H. hampei*-*B. bassiana* interactions. J.

Invertebr. Pathol. 156, 54–72. https://doi.org/10.1016/j.jip.2018.07.008

Wyrebek, M., Huber, C., Sasan, R.K., Bidochka, M.J., 2011. Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. Microbiology 157, 2904–2911. https://doi.org/10.1099/mic.0.051102-0

Table

Table 1 Soil chemical and texture analyses in the plots with diversified and conventional coffee systems in the Experimental Research Station of EPAMIG Patrocínio, Cerrado of Minas Gerais, Brazil

Soil properties	Year	Diversified coffee system			Conventional coffee system		
		B1[2]	B2	B3	B1	B2	B3
pH (H_20)	2019	5.27	4.48	4.96	4.75	4.89	4.72
	2020	4.45	4.59	4.79	5.13	4.84	4.44
	2021	5.00	4.79	4.59	5.78	4.67	4.94
MO[1]	2019	4.30	4.70	4.30	4.96	4.56	4.70
	2020	3.95	3.69	3.95	3.82	4.21	3.82
	2021	3.99	4.12	3.99	3.59	4.12	4.26
Sand (%)	2019	14.8	10.2	9.6	13	10.9	10.4
Silt (%)	2019	17.4	13.8	20.5	18.8	17.8	17.4
Clay (%)	2019	67.8	76	69.9	68.2	71.3	72.2
Classification	2019	very clayey	very clayey	very clayey	very clayey	very clayey	very clayey

[1] MO: organic matter

[2] B: blocks

Figures

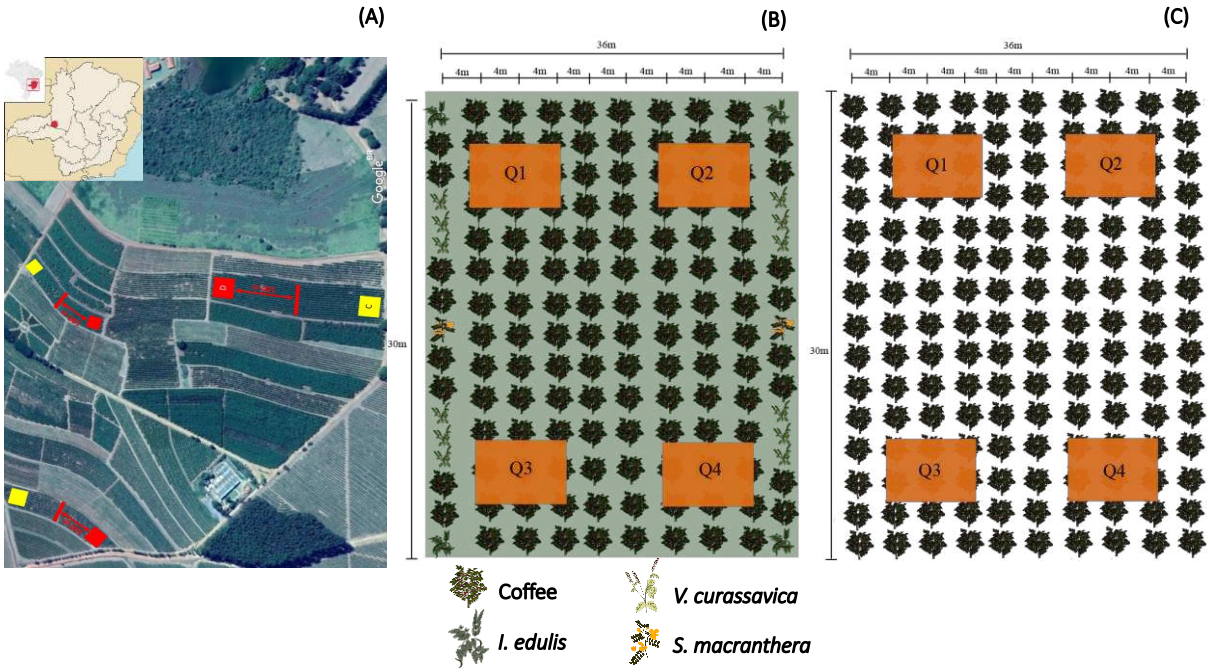

Figure 1. Sketch of the experimental design. (A) Location of plots in the Experimental Research Station of EPAMIG Patrocínio, Cerrado of Minas Gerais, Brazil. Red squares represented plots with diversified coffee system and yellow squares plots with conventional coffee system. (B) Plot with diversified coffee system. (C) plot with conventional coffee system. Each plot measured 1080 m². Brown square delimit four sampling quadrants.

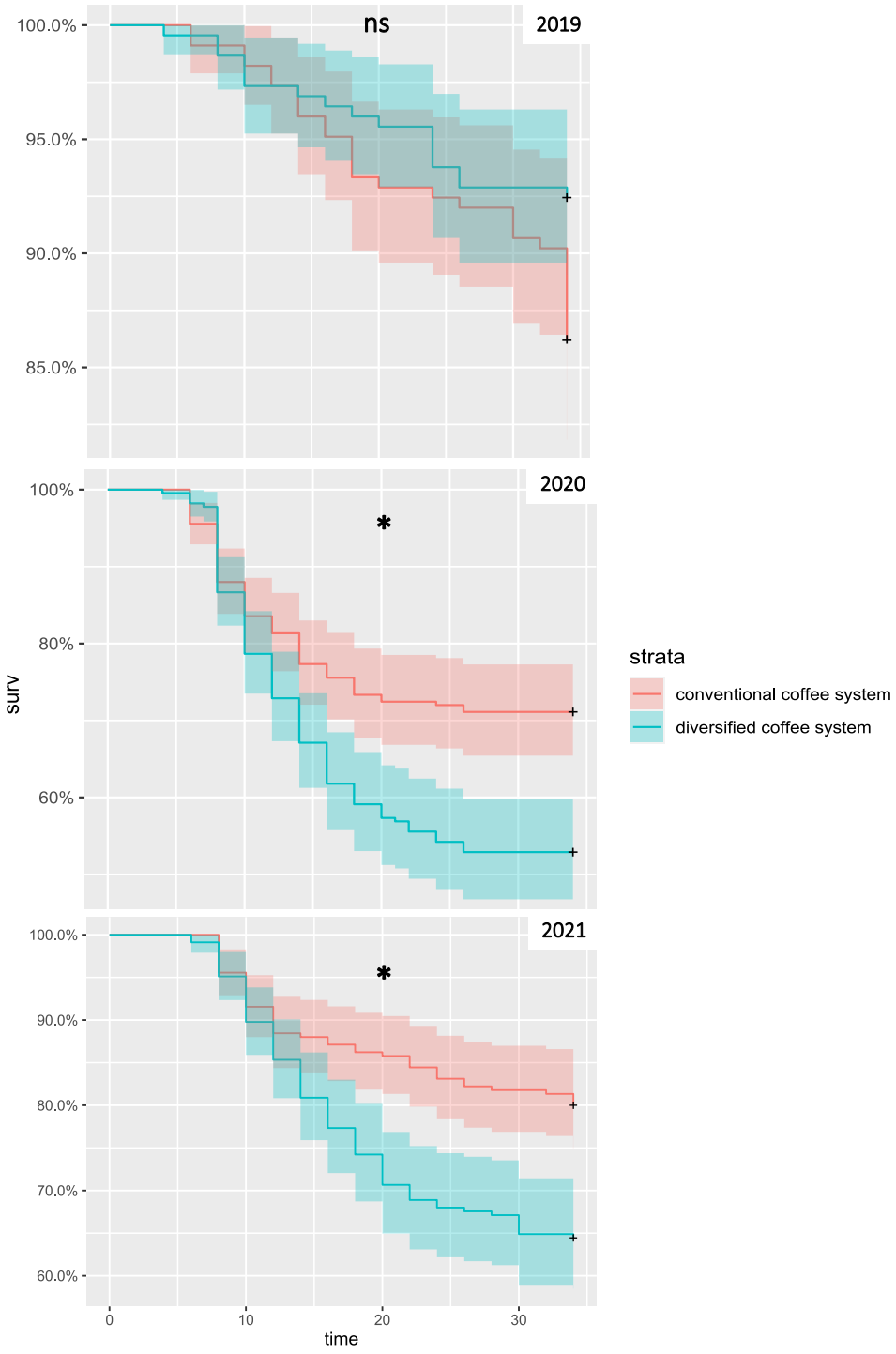

Figure 2 Survivorship of *T. molitor* bait insect larvae in soils from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. 2019: Collect in January 2019 - one month after experiment starts. 2020: Collect in January 2020 - one year after the experiment starts. 2021: Collect in January 2021 - two years after experiment starts. Mortality of bait insects was evaluated for 35 days. Survival analyses are presented in the text. *$p<0.05$, ns - not significant.

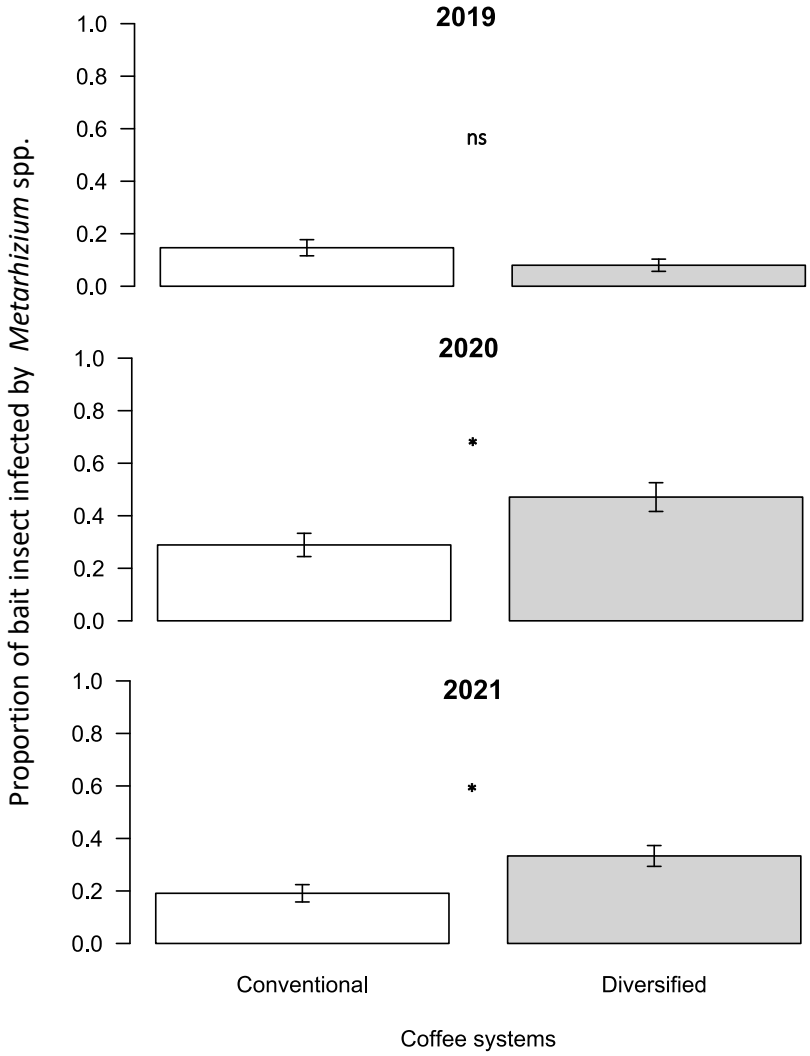

Figure 3 Proportion of bait insect *T. molitor* dead by *Metarhizium* spp. in soil samples (mean ± standard error) from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. 2019: Collect in January 2019 - one month after experiment starts. 2020: Collect in January 2020 - one year after the experiment starts. 2021: Collect in January 2021 - two years after experiment starts. Proportion analyses are presented in the text. *$p<0.05$, ns - not significant.

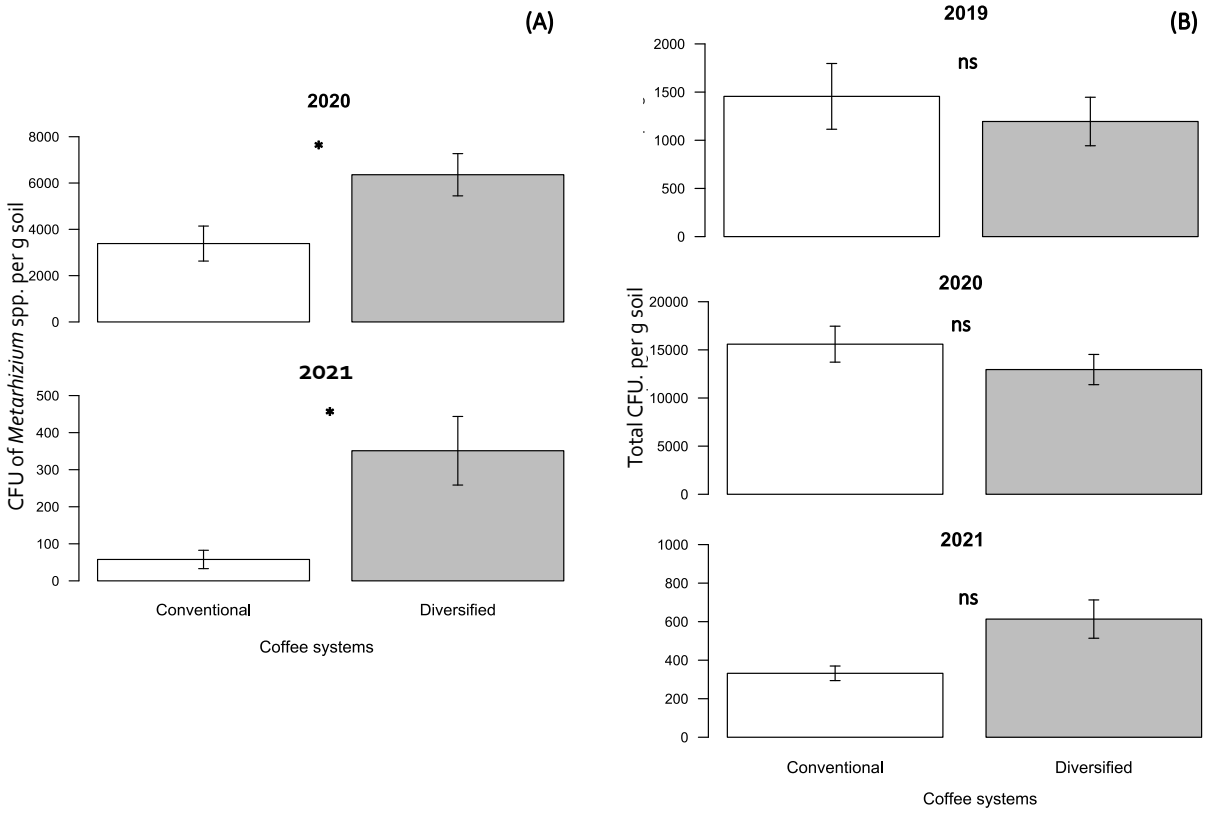

Figure 4 Number of colony-forming units (CFU) (mean ± standard error) in soil from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. (A) Total CFU per g soil (B) CFU of *Metarhizium* spp. per g soil from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. 2019: Collect in January 2019 - one month after experiment starts. 2020: Collect in January 2020 - one year after the experiment starts. 2021: Collect in January 2021 -two years after experiment starts. Density analyses are presented in the text. *$p<0.05$, ns - not significant.

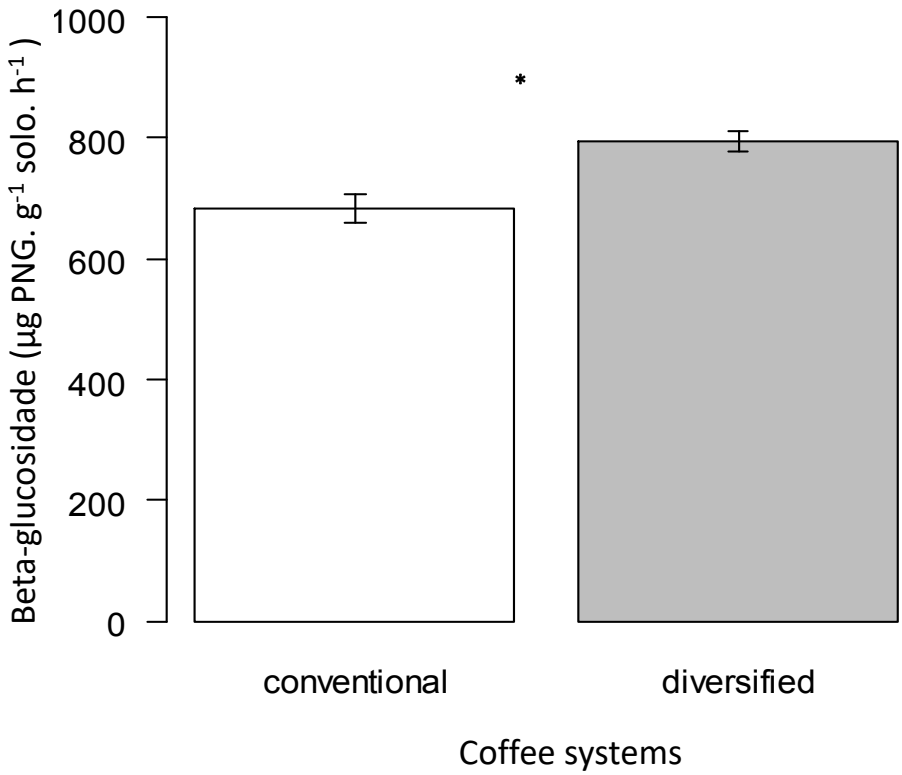

Figure 5 Beta-glycosidase (µg PNG. g^{-1} solo. h^{-1}) (mean ± standard error) in soil from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. Samples collected in January 2021 -two years after experiment starts. Analyses are presented in the text. *$p<0.05$.

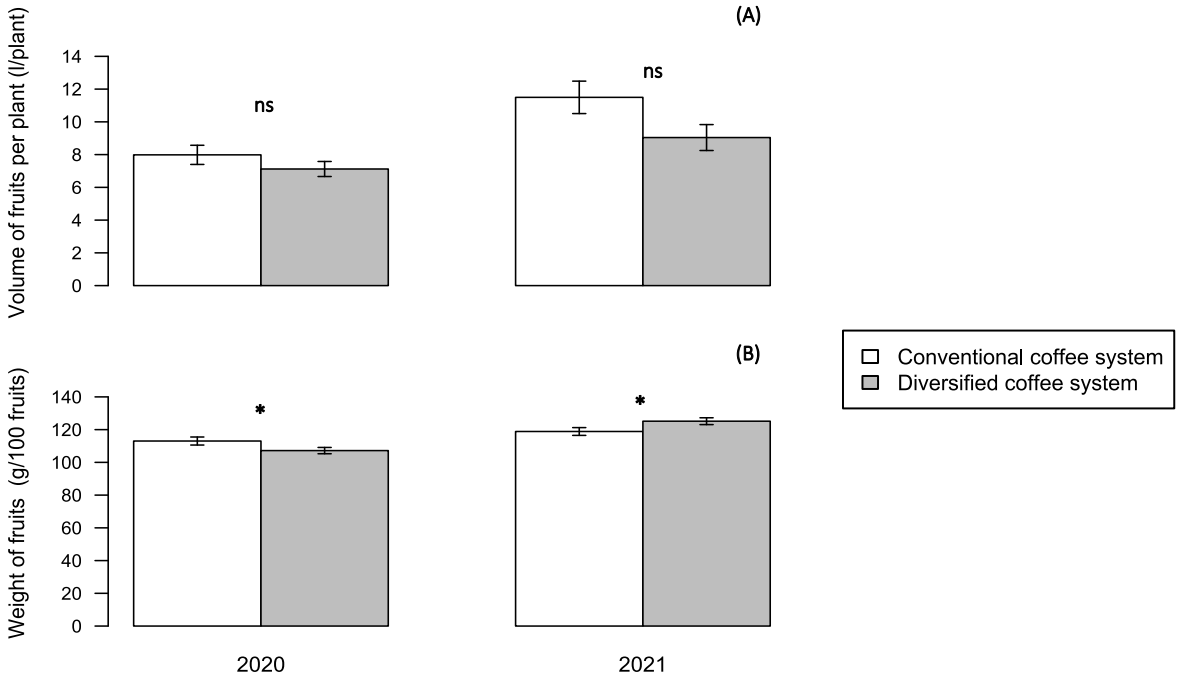

Figure 6. Coffee yield of diversified and conventional coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. (A) Volume of fruits (l/plant). (B) Weight of fruits (g/100 fruits). Analyses are presented in the text. Analyses are presented in the text. *p<0.05, ns - not significant.

Chapter II[1]

Rhizosphere association of *Metarhizium* spp. in diversified coffee system

Abstract

The genus *Metarhizium* (Hypocreales: Clavicipitaceae) is the most abundant entomopathogenic fungi in crop soils. They are known to associate with plant roots, promoting plant growth and protection against pests, therefore, diversified systems with more abundance of plants that *Metarhizium* can associated benefit their maintenance in the soil. We designed a diversified coffee system by association with *Inga edulis* (Fabaceae), *Senna macranthera* (Fabaceae) trees, the perennial bush *Varronia curassavica* (Cordiaceae) and non-crop plants. Our objective was to investigate whether *Metarhizium* spp. colonize these plant roots, and if colonization change during the dry and rainy seasons. We assessed the activity of *Metarhizium* spp. using the bait survival technique with *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. To assess density, we plated soil suspensions on to solid selective media for counts of colony-forming units (CFU). Soil and roots samples from diversified coffee systems had higher activity and density of *Metarhizium* spp. We isolated *Metarhizium* spp. from roots of all plant species in the diversified coffee system, showing that our strategic coffee system improves the maintenance of *Metarhizium* spp. in the coffee crop. Therefore, the diversified coffee system may be an alternative to conventional monoculture, improves activity of *Metarhizium* spp. in coffee roots, which may benefit the crop by promoting plant growth and protection against pests.

KEYWORDS

plant diversification, conservation biological control, soil suppressive, rhizosphere fungi.

[1] Chapter formatted in the norms of Ecology and Evolution

1 | INTRODUCTION

Hypocrealean entomopathogenic fungi are important microbial control agents that cause fungal diseases in almost all orders of insect pests (David, 1967; Meyling & Eilenberg, 2007; Jaramillo et al., 2015; Brunner-Mendoza et al., 2019). They occur naturally in soils (Kepler et al., 2015; Hernández-Domínguez et al., 2016; Sharma et al., 2018; Moreira et al., 2019), where play major roles in the natural regulation arthropods that spend some or all of their life time in the soil (Townsend et al., 1995; Pell et al., 2010). The fungal genus *Metarhizium* (Hypocreales: Clavicipitaceae) has a cosmopolitan distribution in crop soils (Roberts & St. Leger, 2004; Hernández-Domínguez et al., 2016; Sharma et al., 2018; Botelho et al., 2019; Moreira et al., 2019). Several species of this genus are able to associate with rhizospheres (Hu & Leger, 2002; Wyrebek et al., 2011; Behie et al., 2012; Bamisile et al., 2018; Ahmad et al., 2020a). This association can be endophytic, where the fungi develop within plant tissues without causing any noticeable symptoms of disease in the plant (Wilson, 1995; Mantzoukas & Eliopoulos, 2020). Another association is rhizosphere-competence, where the fungi grow in the rhizosphere without colonize plant tissues (Morgan et al., 2005). In both, the fungi increase the root surface area, which improves the absorption of soil nutrients and enhances plant growth (Clark & Zeto, 2000). Endophytic and rhizosphere-competent associations can also promote protection against herbivores (Roberts, 1981; Brotman et al., 2013; Golo et al., 2014; Resquín-Romero et al., 2016; Ríos-Moreno et al., 2016; Garrido-Jurado et al., 2017; McKinnon et al., 2017).

Due the capability of *Metarhizium* spp. to associate with plant roots, the presence of plants benefits their maintenance in soil (St. Leger, 2008; Liao et al., 2014; Iwanicki et al., 2019; Nishi & Sato, 2019). Root exudates may stimulate fungal populations and activities (Baker, 1991; St. Leger, 2008) and improve fungal persistence in the soil (Hu & Leger, 2002; Liao et al., 2014). However, agricultural systems with high plant diversity are not common. Brazil is the largest

producer of coffee in the world (FAOSTAT, 2019), where predominate monocultures and the use of pesticides is the most common control for coffee pests (Venzon, 2021). Cerrado of Minas Gerais, for example, is an important region producing region of arabica coffee in Brazil, responsible for 15% of its production in the country (Conab, 2021), where coffee crops are commonly in extensive areas of monoculture. Monocultures commonly result in proliferation of pests and pathogens, leading to the use of pesticides for pest control. These scenario causes negative effects on soil biota, reducing soil biodiversity (Brown et al., 2020; Winding et al., 2020) and negatively affecting productivity (Nesper et al., 2017). Therefore, increasing plant diversification with plants which *Metarhizium* is able to associate can be a strategy to improve its abundance and ecosystem services in coffee systems. Besides this, diversified systems are known to provide ecosystem services, such as biological control, via attraction and provision of food of natural enemies (Fiedler et al., 2008; Rezende et al., 2014; Amaral et al., 2016; Togni et al., 2019a; 2019b; Venzon et al., 2019)

We designed a diversified coffee system at Cerrado Mineiro with plants that are known to improve conservation biological control of pests by attraction and maintaining predators and parasitoids (Rezende et al., 2014; 2021; Venzon 2021; Amaral et al. 2013): *Inga edulis* (Fabaceae) *Senna macranthera* (Fabaceae) trees, (Marazzi et al., 2013), *Varronia curassavica* (Cordiaceae) perennial bush and non-crop plants. Our objective was to investigate whether *Metarhizium* spp. colonize plant roots of diversified coffee system in the dry and rainy seasons, improving *Metarhizium* spp. activity and density in this soil coffee system. We performed collections in these two seasons, because the soil humidity interferes in the *Metarhizium* conservation in soil (Driesche et al., 2008). Our hypotheses were: (a) soil and roots from diversified coffee system improves activity and density of *Metarhizium* spp.; (b) activity and density of *Metarhizium* spp. are increased in the rainy season; (c) *Metarhizium* spp. has rhizosphere association with *I. edulis, V. curassavica, S. macranthera* and non-crop plant; (d)

coffee roots in diversified systems have more activity and density of *Metarhizium* spp. than conventional coffee roots from conventional systems.

2 | MATERIALS AND METHODS

2.1 | Study area

We installed a diversified coffee system, in December 2018 in a non-irrigated coffee plantation at Experimental Research Station of Agriculture and Livestock Research Enterprise of Minas Gerais (EPAMIG), Patrocínio, Minas Gerais, Brazil (18º59'48''S and 46º59'00''W, elevation ~ 950 m). The region is located in the Cerrado biome, the Brazilian tropical savannah. Patrocínio has Aw climate, according to the Köppen classification, with two distinct seasons, dry winter and rainy. The average annual rainfall is 1,684 mm (Inmet, 2021). The terrain is flat to gently rolling, with an average slope of 3% and the soil is classified as a Red-Yellow Latosol according to Brazilian Soil Classification System (Kamimura et al., 2020).

The experiment was arranged in three blocks of two plots: conventional and diversified coffee system (Figure 1A) and the distance between blocks was at least 500 m. The blocks one and two had *Coffea arabica,* variety "Catuaí Vermelho IAC 99", and the block three had variety "Acaiá IAC 474 - 19". Each plot measured 1080 m² (30 x 36 m) (Figure 1) separated from each other by 200 m. The diversified coffee system plots had four *I. edulis* trees, 12 *V. curassavica* perennial bushes and two *S. macranthera* trees (Figure 1B). Non-crop plants were maintained between rows and mechanic suppression was done whenever the height exceeded 50 cm. In June, before coffee harvest, the non-crop plants were mechanic suppressed up to 10 cm to facilitate post-harvesting treatment in the crop. No pesticides were used in the diversified coffee system, but agronomic practices with mineral fertilization were maintained under standard

coffee management of the Cerrado (Appendix 1). The plots under coffee conventional system were arranged only with coffee plants (Figure 1C). These plots were conventionally managed, implying that fertilizers and pesticides are used under standard of coffee management the Cerrado (Appendix 1).

2.2 | Collection of roots and soil samples

We collected soil and roots in the dry (September 2020) and in the rainy season (January 2021), to investigate whether these seasons influence entomopathogenic fungi. In each season, we collected roots of eight coffee plants, four *I. edulis* and *V. curassavica* plants, two *S. macranthera*. In the dry season, we collected four *Gnaphalium spicatum, Conyza bonariensis* and *Solanum Americanum*. In the rainy season, we collected four *Ageratum conyzoides, Bidens pilosa* and *Sida cordifolia*. We collected these species because they were the most abundant non-crop plants species in the experimental area in each season. In each conventional coffee plot, we collected eight coffee plant roots. We collected the roots with hoe, removed the soil excess and placed them in plastic bags. We collected the entire root system of non-crop plants and lateral roots coming from large roots of coffee, *V. curassavica*, *S. macranthera* and *I. edulis*. For each non-crop plant, we take pictures and collected a foliage sample for further plant identification. The roots were refrigerated at 5°C for five days, when we finished processing them in the laboratory.

In both root collection, we also took soil samples near each plant sampled to evaluated the activity and abundance of *Metarhizium* spp. in soil, without roots. We took 18 soil samples in the diversified coffee system plot and 8 soil samples in the conventional coffee system plot, with a core soil sampler to 20 cm depth. We did not take soil samples near the non-crop plants, because it was impossible collect soil without roots.

2.3 | Activity of *Metarhizium* spp.

Healthy mealworm larvae *Tenebrio molitor* (Coleoptera: Tenebrionidae) were used as bait insects to assesses the entomopathogenic ability *Metarhizium* present in the soil and roots in killing the bait insects (Moreira et al., 2019). The insect larvae were obtained from a stock rearing maintained on wheat bran chayote, carrot and potato at Laboratory of Entomology in EPAMIG, in Viçosa. Each root sample were shaken in a standardized manner to remove non-rhizosphere soil and the rhizosphere soil was defined as the soil still adhering to the roots after shaking. Five g from each root sample was placed into a 200 ml transparent plastic pot sterilized in UV light for 15 minutes and covered with a plastic lid that contained moistened filter paper. From each soil sample, a subsample of soil was transferred to a 200 ml transparent plastic pot sterilized in UV light for 15 minutes and covered with a plastic lid with four holes for ventilation.

We selected five two-month-old larvae of similar size (1.3–1.5 cm) and introduced them to each plastic pot containing root or soil. The pots with root were incubated in a climate room at 25±5 ºC in complete darkness and the pots with soil were incubated in a climate room at 25±5 ºC and 12:12 (L:D). We inverted and left upside down the pots with soil to force the insects to traverse the soil during the first 10 days. We check the mortality of *T. molitor* every two days, inspecting the pots with a sterile tweezer, for 35 days. The pots were open next to a flam for a sterile microenvironment and the live larvae were kept in the pots for later assessments. The dead larvae were sterilized by immersion in 70% alcohol, 5% sodium hypochlorite, rinsed in distillated water and dried on sterile filter paper. After that, the larvae were incubated in a sterile microtube with a moistened cotton wool with distilled water, kept in a climate chamber (25±5 ºC) to promote fungal growth. We inspected the incubated larvae daily for the presence of external fungi growth and all fungi detected were isolated in PDA with 0.05

g l^{-1} chloramphenicol and incubated at 25 ºC. We prepared slides for morphological identification in microscopy (40 x objective) with the isolates reproductive structures. We also calculated the proportion of bait insect infected by *Metarhizium* per soil sample and the survival of bait insect.

2.4 | Density of *Metarhizium spp.*

We counted colony-forming units (CFU) of *Metarhizium* spp. in soil and roots samples. The roots were shaken by hand to remove soil particles and the soil directly adhered to the root after that, representative the rhizosphere. One gram of roots was ground with mortar and pestle and then diluted in 5 ml of sterile solution of Tween 0.01% in a Falcon® tube (15 ml). Tubes were rotated for one hour in a rotary shaker at 150 rpm (Lacey, 2012), vortexed for 15 s and then platted on to selective culture media. We used a selective medium with 10 g peptone, 20 g dextrose, 15 g agar, and 1 l distilled water. After sterilizing the medium, we added 0.05 g/l of cycloheximide and tetracycline, 0.6 g/l streptomycin and 0.175 g/l CTAB (cetyltrimethylammonium bromide) (Kepler et al., 2015). One hundred microliters from each suspension were plated in three Petri dishes (9 cm diameter), and spread with a sterile Drigalski spatula. Plates were kept in the dark at 25 °C for 14 days until CFU counts could be done. For soil samples, 5g of the soil were diluted in 45 ml of sterile solution of Tween 0.01% and we performed the same procedure used for root. The concentration of CFU per gram of root was made by multiplying the number of CFU (medium from three Petri dishes) by 50, as 100 µl of the root suspension has 0.02 g root. The concentration of CFU per gram of soil was made by multiplying the number of CFU (medium from three Petri dishes) by 100, as 100 µl of the soil suspension has 0.01 g soil.

2.5 | Statistical analyses

Analyses were performed using the R statistical system (R Core Team, 2017). We used systems (conventional and diversified coffee systems), plants (*I. edulis, V. curassavica, S. macranthera* and non-crop plants) and seasons (dry and rainy season) as explanatory variables to investigate how diversified coffee system affect density and activity of *Metarhizium* spp. in the rhizosphere. Survival analyses was carried out solely the dead insects with *Metarhizium* spp. signs of infection to test time to death of *T. molitor* larvae bait in plant roots. We added the R software package "frailtypack" (Rondeau et al., 2019) in the models with gamma distributions, as a penalization to fit a joint frailty model of the five insects in same sample. Analyses were ANOVA with χ^2 tests. We used generalized linear model (GLM) adjusted to binomial distribution and analyzed by ANOVA with χ^2 tests to examine the proportion of *Metarhizium* spp. found in bait insects (Crawley, 2015). To test the numbers of total and *Metarhizium* spp. CFU, we GLM adjusted to Poisson distribution and analyzed by ANOVA with χ^2 tests.

3 | RESULTS

3.1 | Activity of *Metarhizium*

The survival of bait insects was used to measure the activity of *Metarhizium* spp. There was interaction of coffee systems (χ^2=35.67, p<0.001), seasons (χ^2=82.29, p<0.001) and plant species (χ^2=77.03, p<0.001) in the survival of bait insects. In the dry season, the bait insect survival was similar between coffee systems (t=0.003, p=1.000, Figure 2A). However, in the

rainy season the bait insects died faster in the diversified than in the conventional coffee system roots (t=4.29, p<0.001, Figure 2B). The survival in conventional coffee system was similar between the seasons (t=2.270, p=0.1058). However, the survival in diversified coffee system decreased in the rainy season (t=7.95, p<0.001). When we evaluated the plant roots separately, there was no difference between coffee roots of conventional and diversified systems (dry season: t=2.76, p=0.29, Figure 3A; rainy season: t=2.21, p=0.68, Figure 3B). Bait insects died faster in roots of *I. edulis* than in coffee roots from conventional system, in both seasons (Tables 1, 2 and Figure 3). Furthermore, bait insect survival was lower in roots of *I. edulis, V. curassavica, S. macranthera* and *A. conyzoides* than conventional coffee in the rainy season (Table 2, Figure 3). Also in the rainy season, bait insect survival was lower in roots of I. edulis and S. macranthera than in roots of coffee from diversified system. All other pairwise comparisons are in Tables 1, 2 and Figure 3. In soil samples, bait insects died faster in soil from diversified than conventional coffee system (dry season: t=4.67, p<0.001, Figure 2A; rainy season: t= 3.82, p=0.0034, Figure 2B).

3.2 | Proportion of *Metarhizium* spp.

There was interaction of coffee systems (χ^2=36.43, p<0.001) and seasons (χ^2=39.23, p<0.001), but not of plant species (χ^2=3.17, p=0.17) in the proportion of bait insects infected by *Metarhizium* spp. in roots. In both seasons, the proportion was higher in the roots of diversified than conventional coffee system (dry season: z=2.54, p=0.04, Figure 4A; rainy season: z=2.54, p=0.04, Figure 4B). There was no difference between the seasons in both conventional and diversified roots (z=2.25, p=0.10). In soil, the proportion of bait insects infected by *Metarhizium* spp. was higher in diversified than conventional coffee system (dry season: z=3.62, p=0.0071, Figure 4A; rainy season: z=2.41, p=0.0018, Figure 4B).

3.3 | Density of *Metarhizium* spp.

Metarhizium spp. density was influenced by the coffee systems (χ^2=9236.70, p<0.001), the seasons (χ^2=9366.10, p<0.001) and plant species (χ^2=9444.10, p<0.001). In the dry season, the number of *Metarhizium* spp. CFU was similar between diversified and conventional coffee system roots (z=1.41, p=0.49, Figure 5A). However, in the rainy season, the number of *Metarhizium* spp. CFU was higher in the diversified than conventional coffee system roots (z=2.62, p=0.04, Figure 5B). Density of *Metarhizium* spp. in roots of conventional system was similar between the seasons (z=0.030, p=1.00). However, the density in roots of diversified system increased in rainy season (z=4.39, p<0.001). There was no difference between density of *Metarhizium* spp. in coffee roots in the diversified and in conventional system in the dry season (z=2.08, p=0.29, Figure 6A). However, in the rainy season, the abundance of *Metarhizium* spp. was higher in diversified than conventional coffee roots (z=3.41, p=0.01, Figure 6B). Besides, *I. edulis* had more CFU of *Metarhizium* spp. than conventional coffee in both seasons (dry: z=3.18, p=0.03, Figure 6A, rainy: z=3.694, p=0.005, Figure 6B). *S. cordifolia* also had more CFU of *Metarhizium* spp. than conventional coffee (z=4.40, p<0.001, Figure 6B). All other pairwise comparisons are in Tables 3, 4 and Figure 6. In the soil, the density of *Metarhizium* spp. in the dry season was similar between diversified and conventional coffee system (z=2.14, p=0.34, Figure 5A). However, in the rainy season, the density was higher in diversified and conventional coffee system (z=2.88, p=0.003, Figure 5B).

4 | DISCUSSION

Our results showed that diversified coffee system is more favorable to maintenance of *Metarhizium* spp. than conventional coffee system, in both seasons where climatic conditions

are more favorable or more severe for fungi development. Diversified coffee system is known to correlate positively with abundance and persistence of propagules of *Metarhizium* spp. (Moreira et al., 2019). It can be explained by the ability of diversified systems promote shaded soil (Jose, 2009), increasing soil humidity within the plots, which improve fungi processes such as sporulation, germination and infection of hosts (Driesche et al., 2008). Additionally, the presence of more plants in diversified systems promotes more root exudates in soil, which may stimulate microbial populations and their activities (Baker, 1991; St. Leger, 2008).

Metarhizium spp. can persist in soil when host insects are absent aboveground (Botelho et al., 2019; Moreira et al., 2019; Pell et al., 2010). Due their versatile lifestyle, they can persist in soil as saprotrophs (Hu and St Leger, 2002) or as rhizosphere colonizers (Sasan and Bidochka, 2012). *Metarhizium* spp. colonize different plants, such as bean *Phaseolus vulgaris*, switchgrass *Panicum virgatum* (Behie et al., 2012), perennial herbs, shrubs, coniferous trees (Fisher et al., 2011), oilseed rape *Brassica napus*, winter wheat *Triticum aestivum*, grass pasture (Keyser et al., 2015) sugarcane *Saccharum* spp. (Iwanick et al., 2019), potato (Krell et al., 2018) and many others. However, they generally colonize monocots better than dicots, which indicates some level of plant host specificity, but it is unknown whether the association is dictated by the plant and/or the fungus (Moonjely & Bidochka, 2019). We collected roots only from dicots, because they are the most abundant species in our experimental area and we isolated *Metarhizium* spp. in all dicots of diversified coffee system.

The maintenance of fungi in soil depends of abiotic factors, because their germination, infection, and sporulation require soil moisture (Driesche et al., 2008) and low solar radiation (de Castro et al., 2013). We evaluated density and activity of *Metarhizium* spp. in plant roots in two seasons: dry - without rain in the last four months before root collect - and rainy - with 126 mm of precipitation in the month of root collection (Inmet, 2021). In general, the activity and density of *Metarhizium* spp. in the diversified coffee system was higher in the rainy season than

in the dry. However, the season did not influence the activity and density of *Metarhizium* spp. in the conventional coffee system and they are always lower than in the diversified system. Despite the increase in soil moisture caused by rain, others factors such as use of pesticides and full-sun managed soil in the conventional coffee system causes negative effects on soil biota (Brown et al., 2020). Applications of insecticides, fungicides and herbicides could impact entomopathogenic fungi in both soil and foliar environment by killing or inhibiting fungal propagules or removing hosts (Wekesa et al., 2008). Solar radiation is detrimental to the persistence of the fungi (de Castro et al., 2013). Therefore, even when soil moisture was higher, there was no improve *Metarhizium* spp. on conventional coffee system compared to dry season.

Metarhizium spp. had more activity in roots of *I. edulis* and *S. macranthera* than on roots from coffee, either from conventional or from diversified systems. These species are both leguminous and others studies showed *Metarhizium* spp. more frequent in leguminous plants (Randhawa et al., 2018; Ahmad et al., 2020b). It may be due to the quantity or quality of root exudates, which may be better in leguminous (Biate et al., 2015). Besides*, I. edulis* and *S. cordifolia* roots had more density of *Metarhizium* spp. than conventional coffee roots. These results suggest that strategic plant diversification is favorable to conservation of *Metarhizium* spp. in coffee crops, mainly due to *I. edulis*, *S. macranthera* and *S. cordifolia* roots maintained in the diversified coffee system had more *Metarhizium* spp. than coffee plants. The root surface may provide a nutrient base for fungi and the fungi may assist the plant by solubilizing inorganic nutrients or as biocontrol agents against plant pathogens (St. Leger, 2008). Besides, the root exudates may stimulate or inhibit microbial populations and their activities (Baker, 1991; St. Leger, 2008). Also, diversified coffee roots had more CFU of *Metarhizium* spp. than conventional coffee roots in the rainy season, indicating that in diversified coffee system is more common to find *Metarhizium* spp. in the rhizosphere of coffee plants compared to conventional coffee system. Therefore, the benefits of *Metarhizium* roots association, such as

plant growth and protection against pests (Behie et al., 2012; Behie & Bidochka, 2014; Jaber & Araj, 2018) is more probably in coffee roots of diversified than conventional coffee system.

The presence of *Metarhizium* spp. in the plant roots of diversified coffee system showed that our strategic coffee system improves the maintenance of *Metarhizium* spp. in the coffee crops. The associations of *Metarhizium* spp. with plant roots are known to improve fungal persistence in soils, in the absence of an insect hosts (Hu & Leger, 2002), playing important role in the persistence and stability of *Metarhizium* spp. in the field (Liao et al., 2014). Therefore, diversified coffee system may be an alternative to conventional monoculture, by improving the activity of *Metarhizium* spp. in coffee roots, which can benefit the crop such as promote plant growth and protection against pests (Behie et al., 2012; Behie & Bidochka, 2014; Jaber & Araj, 2018). Further studies are necessary to investigate these benefits in coffee crop.

ACKNOWLEDGMENTS

The present study was supported by ‘Conselho Nacional de Desenvolvimento Científico e Tecnológico’ (CNPq), ‘Fundação de Amparo a Pesquisa de Minas Gerais’ (FAPEMIG), 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil' (CAPES) - Finance Code 001 and Consórcio Brasileiro de Pesquisa e Desenvolvimento do Café (CBP&D-Café). We are grateful Jaime Silva, Diego Vilela, Adalberto (Nenzinho), Ronaldo Gaspar, Hélio Bernardes and the coffee harvest workers of EPAMIG - Patrocínio for all their support in carrying out the field experiments. We thank Leila Nascimento, Marcela Caixeta, Keminy Bautz, Elenir Queiroz, Walysson Mendes, Eduardo Costantin and Verônica Fialho from the laboratory of Insect-Microorganism Interactions, UFV, for their help with laboratory protocols. We also thank Jéssica Martins for her support in preparing the sketch of the experimental design.

REFERENCES

Ahmad, I., Jiménez-Gasco, M. M., Luthe, D. S., Shakeel, S. N., & Barbercheck, M. E. (2020a). Endophytic *Metarhizium robertsii* promotes maize growth, suppresses insect growth, and alters plant defense gene expression. *Biological Control,* 144, 104167. https://doi.org/10.1016/j.biocontrol.2019.104167.

Ahmad, I, Jiménez-Gasco, M. M., Luthe, D. S., & Barbercheck, M. E. (2020b). Systemic Colonization by *Metarhizium robertsii* Enhances Cover Crop Growth. *Journal of Fungi* 6, 64. https://doi.org/10.3390/jof6020064

Amaral, D. S. S. L., Venzon, M., Duarte, M. V. A., Sousa, F. F., Pallini, A., & Harwood, J. D. (2013). Non-crop vegetation associated with chili pepper agroecosystems promote the abundance and survival of aphid predators. *Biological Control,* 64, 338–346. https://doi.org/10.1016/j.biocontrol.2012.12.006.

Amaral, D. S. S. L., Venzon, M., Helder, H., Sujii, E. R., Schmidt, J. M., & Harwood, J. D. (2016). Non-crop plant communities conserve spider populations in chili pepper agroecosystems. *Biological Control,* 103, 69–77. https://doi.org/10.1016/j.biocontrol.2016.07.007.

Baker, R. (1991). Diversity in biological control. *Crop Protection,* 10, 85–94. https://doi.org/10.1016/0261-2194(91)90054-U.

Bamisile, B. S., Dash, C. K., Akutse, K. S., Keppanan, R., & Wang, L. (2018). Fungal endophytes: Beyond herbivore management. *Frontiers in Microbiology,* 9, 544. https://doi.org/10.3389/fmicb.2018.00544.

Behie, S. W., & Bidochka, M. J. (2014). Nutrient transfer in plant–fungal symbioses. *Trends Plant Science*19, 734–740. https://doi.org/10.1016/j.tplants.2014.06.007.

Behie, S. W., Zelisko, P. M., & Bidochka, M. J. (2012). Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. *Science,* 336, 1576–1577. https://doi.org/10.1126/science.1222289.

Biate, D. L., Kumari, A., Annapurna, K., Kumar, L. V., Ramadoss, D., Reddy, K. K., Naik, S., 2015. Legume root exudates: Their role in symbiotic interactions, in: Arora, N.K. (Ed). *Plant Microbes Symbiosis: Applied Facets*. Springer, India. pp. 259–271.

Botelho, A. B. R. Z., Alves-Pereira, A., Prado, R. C., Zucchi, M. I., & Delalibera Júnior, I. (2019). *Metarhizium* species in soil from Brazilian biomes: a study of diversity, distribution, and association with natural and agricultural environments. *Fungal Ecology,* 41, 289–300. https://doi.org/10.1016/j.funeco.2019.07.004.

Brotman, Y., Landau, U., Cuadros-Inostroza, Á., Takayuki, T., Fernie, A. R., Chet, I., ... Willmitzer, L. (2013). *Trichoderma*-plant root colonization: escaping early plant defense responses and activation of the antioxidant machinery for saline stress tolerance. *PLoS Pathogens,* 9, e1003221. https://doi.org/10.1371/journal.ppat.1003221.

Brown, G., Cooper, M., and Kobayashi, M. (2020). "Threats to soil biodiversity – global and regional trends," in *State of knowledge of soil biodiversity: Status, challenges and potentialities*, eds. FAO, ITPS, GSBI, CBD, and EC (Roma: FAO), 191–262.

Brunner-Mendoza, C., Reyes-Montes, M. del R., Moonjely, S., Bidochka, M. J., and Toriello, C. (2019). A review on the genus *Metarhizium* as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico. *Biocontrol Science and Technology,* 29, 83–102. https://doi.org/10.1080/09583157.2018.1531111.

Clark, R. B., & Zeto, S. K. (2000). Mineral acquisition by arbuscular mycorrhizal plants. *Journal of Plant Nutrition,* 23, 867–902. https://doi.org/10.1080/01904160009382068.

Conab (2021). Acompanhamento da Safra Brasileira: Café. *Companhia. Nacional Abastecimento.* Available at: https://www.conab.gov.br/index.php/info-agro/safras/cafe [Accessed September 20, 2021].

Crawley, M. J. (2015). *Statistics: An introduction using R*. 2nd ed. , ed. M. J. Crawley United kingdom: John Wiley & Sons, Ltd

David, W. A. L. (1967). "The physiology of the insect integument in relation to the invasion of pathogens," in *Insects and physiology*, ed. J. E. Beament, J.W.L.; Treherne (Edinburgh, &c., Oliver & Boyd.), 17–35.

de Castro, T. R., Wekesa, V. W., Moral, R. D. A., Demétrio, C. G. B., Delalibera, I., & Klingen, I. (2013). The effects of photoperiod and light intensity on the sporulation of brazilian and norwegian isolates of *Neozygites floridana*. *Journal of Invertebrate Pathology,* 114, 230–233. https://doi.org/10.1016/j.jip.2013.08.004.

Driesche, R. Van, Hoddle, M., & Center, T. (2008). *Control of pests and weeds by natural enemies: An introduction to biological control*. 1st ed. , eds. R. Van Driesche, M. Hoddle, and T. Center Oxford UK: Blackwell publishing Ltd . https://doi.org/10.1653/024.092.0237.

FAOSTAT, 2019. Food and Agriculture Organization of the United Nations. URL http://faostat.fao.org/faostat. (accessed 9.20.21).

Fiedler, A. K., Landis, D. A., & Wratten, S. D. (2008). Maximizing ecosystem services from conservation biological control: The role of habitat management. *Biological Control,* 45, 254–271. . https://doi.org/10.1016/j.biocontrol.2007.12.009.

Fisher, J. J., Rehner, S. A., & Bruck, D. J. (2011). Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. *Journal of*

Invertebrate Pathology, 106, 289–295. https://doi.org/10.1016/j.jip.2010.11.001.

Garrido-Jurado, I., Resquín-Romero, G., Amarilla, S. P., Ríos-Moreno, A., Carrasco, L., and Quesada-Moraga, E. (2017). Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). *Journal of Pest Science,* 90, 319–330. https://doi.org/10.1007/s10340-016-0767-2.

Golo, S., Gardner, D. R., Grilley, M. M., Takemoto, J. Y., Krasnoff, S. B., Pires, M. S., ... Roberts, D. W. (2014). Production of destruxins from *Metarhizium* spp . fungi in artificial medium and in endophytically colonized cowpea plants. *PLoS One,* 9, e104946. https://doi.org/10.1371/journal.pone.0104946.

Hernández-Domínguez, C., Cerroblanco-Baxcajay, M. L., Alvarado-Aragón, L. U., Hernández-López, G., & Guzmán-Franco, A. W. (2016). Comparison of the relative efficacy of an insect baiting method and selective media for diversity studies of *Metarhizium* species in the soil. *Biocontrol Science and Technology,* 26, 707–717. https://doi.org/10.1080/09583157.2016.1152458.

Hu, G., & Leger, R. J. S. (2002). Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Applied and Environmental Microbiology,* 68, 6383–6387. https://doi.org/10.1128/AEM.68.12.6383.

Inmet (2021). Instituto Nacional de Metereologia. Available at: https://clima.inmet.gov.br/prec [Accessed July 8, 2021].

Iwanicki, N. S., Pereira, A. A., Botelho, A. B. R. Z., Rezende, J. M., Moral, R. de A., Zucchi, M. I., ... Delalibera Júnior, I. (2019). Monitoring of the field application of *Metarhizium anisopliae* in Brazil revealed high molecular diversity of *Metarhizium* spp in insects, soil and sugarcane roots. *Scientific Reports,* 9, 1–12. https://doi.org/10.1038/s41598-019-

38594-8.

Jaber, L. R., & Araj, S. E. (2018). Interactions among endophytic fungal entomopathogens (Ascomycota: Hypocreales), the green peach aphid *Myzus persicae* Sulzer (Homoptera: Aphididae), and the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae). *Biological Control,* 116, 53–61. https://doi.org/10.1016/j.biocontrol.2017.04.005.

Jaramillo, J. L., Montoya, E. C., Benavides, P., & Góngora B, C. E. (2015). *Beauveria bassiana* y *Metarhizium anisopliae* para el control de broca del café en frutos del suelo. *Revista Colombiana de Entomología,* 41, 95–104.

Jose, S. (2009). Agroforestry for ecosystem services and environmental benefits: An overview. *Agroforestry Systems,* 76, 1–10. https://doi.org/10.1007/s10457-009-9229-7.

Kamimura, K. M., Dias Júnior, M. de S., de Oliveira, M. S., Dos Santos, G. R., Guimarães, P. T. G., & Ajayi, A. E. (2020). Spatial variability of precompression stress and volumetric water content of a red-yellow latosol (Oxisol). *Bioscience Journal,* 36, 142–151. https://doi.org/10.14393/BJ-v36n1a2020-42121.

Kepler, R. M., Ugine, T. A., Maul, J. E., Cavigelli, M. A., & Rehner, S. A. (2015). Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. *Environmental Microbiology,* 17, 2791–2804. https://doi.org/10.1111/1462-2920.12778.

Keyser, C. A., De Fine Licht, H. H., Steinwender, B. M., & Meyling, N. V. (2015). Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. *BMC Microbiology,* 15, 249. https://doi.org/10.1186/s12866-015-0589-z.

Krell, V., Jakobs-Schoenwandt, D., Vidal, S., & Patel, A. V (2018). Cellulase enhances endophytism of encapsulated *Metarhizium brunneum* in potato plants. *Fungal Biology,* 122, 373–378 https://doi.org/10.1016/j.funbio.2018.03.002.

Lacey, L. A. (2012). *Manual of techniques in invertebrate pathology*. 2nd ed. , ed. L. A. Lacey Yakima, Washington, EUA: Elsevier Ltd.

Liao, X., O'Brien, T. R., Fang, W., & St. Leger, R. J. (2014). The plant beneficial effects of *Metarhizium* species correlate with their association with roots. *Applied Microbiology and Biotechnology,* 98, 7089–7096. https://doi.org/10.1007/s00253-014-5788-2.

Mantzoukas, S., & Eliopoulos, P. A. (2020). Endophytic entomopathogenic fungi: A valuable biological control tool against plant pests. *Applied Sciences,* 10, 360. https://doi.org/10.3390/app10010360.

Marazzi, B., Conti, E., Sanderson, M. J., Mcmahon, M. M., & Bronstein, J. L. (2013). Diversity and evolution of a trait mediating ant-plant interactions: Insights from extrafloral nectaries in *Senna* (Leguminosae). *Annals of Botany,* 111, 1263–1275. https://doi.org/10.1093/aob/mcs226.

McKinnon, A. C., Saari, S., Moran-Diez, M. E., Meyling, N. V., Raad, M., & Glare, T. R. (2017). *Beauveria bassiana* as an endophyte: a critical review on associated methodology and biocontrol potential. *BioControl,* 62, 1–17. https://doi.org/10.1007/s10526-016-9769-5.

Meyling, N. V, & Eilenberg, J. (2007). Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems : Potential for conservation biological control. *Biological Control,* 43, 145–155. https://doi.org/10.1016/j.biocontrol.2007.07.007.

Moonjely, S., & Bidochka, M. J. (2019). Generalist and specialist *Metarhizium* insect pathogens retain ancestral ability to colonize plant roots. *Fungal Ecology,* 41, 209–217. https://doi.org/10.1016/j.funeco.2019.06.004.

Moreira, C. C., Celestino, D., Guerra, T., Cardoso, I. M., & Elliot, S. L. (2019). Agroforestry coffee soils increase the insect-suppressive potential offered by entomopathogenic fungi over full-sun soils : A case proposing a "bait survival technique." *Ecology and. Evolution,* 9, 10777–10787. https://doi.org/10.1002/ece3.5598.

Morgan, J. A. W., Bending, G. D., & White, P. J. (2005). Biological costs and benefits to plant-microbe interactions in the rhizosphere. *Journal of Experimental Botany* 56, 1729–1739. https://doi.org/10.1093/jxb/eri205.

Nesper, M., Kueffer, C., Krishnan, S., Kushalappa, C. G., Ghazoul, J., 2017. Shade tree diversity enhances coffee production and quality in agroforestry systems in the Western Ghats. *Agriculture Ecosystems & Environment*. 247, 172–181. https://doi.org/10.1016/j.agee.2017.06.024.

Nishi, O., & Sato, H. (2019). Isolation of *Metarhizium* spp . from rhizosphere soils of wild plants reflects fungal diversity in soil but not plant specificity. *Mycology*, 10, 22–31. https://doi.org/10.1080/21501203.2018.1524799

Pell, J. K., Hannam, J. J., & Steinkraus, D. C. (2010). Conservation biological control using fungal entomopathogens. *BioControl,* 55, 187–198. https://doi.org/10.1007/s10526-009-9245-6.

Randhawa, P. K., Mullen, C., & Barbercheck, M. (2018). Plant identity, but not diversity, and agroecosystem characteristics affect the occurrence of *M. robertsii* in an organic cropping system. *Biological Control*, 124, 18-29. https://doi.org/10.1016/j.biocontrol.2018.06.001

Resquín-Romero, G., Garrido-Jurado, I., Delso, C., Ríos-Moreno, A., & Quesada-Moraga, E. (2016). Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. *Journal of Invertebrate Pathology,* 136, 23–31. https://doi.org/10.1016/j.jip.2016.03.003.

Rezende, M. Q., Venzon, M., Santos, P. S., Cardoso, I. M., & Janssen, A. (2021). Extrafloral nectary-bearing leguminous trees enhance pest control and increase fruit weight in associated coffee plants. *Agriculture Ecosystems & Environment,* 319, 107538. https://doi.org/10.1016/j.agee.2021.107538

Rezende, M. Q., Venzon, M., Perez, A. L., Cardoso, I. M., & Janssen, A. (2014). Extrafloral nectaries of associated trees can enhance natural pest control. *Agriculture Ecosystems & Environment,* 188, 198–203. https://doi.org/10.1016/j.agee.2014.02.024.

Ríos-Moreno, A., Garrido-Jurado, I., Resquín-Romero, G., Arroyo-Manzanares, N., Arce, L., & Quesada-Moraga, E. (2016). Destruxin A production by *Metarhizium brunneum* strains during transient endophytic colonisation of *Solanum tuberosum*. *Biocontrol Science and Technology,* 26, 1574–1585. https://doi.org/10.1080/09583157.2016.1223274.

Roberts, D. W. (1981). "Toxins of entomopathogenic fungi," in *Microbial Control of Pests and Plant Disease 1970–1980*, ed. H. D. Burges (London: Academic Press), 441–463.

Roberts, D. W., & St. Leger, R. J. (2004). *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects. *Advances in Applied Microbiology,* 54, 1–70. https://doi.org/10.1016/S0065-2164(04)54001-7.

Rondeau, V., Gonzalez, J. R., Yassin Mazroui, A. Mauguen, A. D., Laurent, A., Lopez, M., & Rondeau, M. V. (2019). Package 'frailtypack'.

Sasan, R. K., Bidochka, M. J., 2012. The insect-pathogenic fungus *Metarhizium robertsii*

(Clavicipitaceae) is also an endophyte that stimulates plant root development. *American Journal of Botany* 99, 101–107. https://doi.org/10.3732/ajb.1100136

Sharma, L., Oliveira, I., Torres, L., & Marques, G. (2018). Entomopathogenic fungi in Portuguese vineyards soils: suggesting a 'Galleria-Tenebrio-bait method' as bait- insects *Galleria* and *Tenebrio* significantly underestimate the respective recoveries of *Metarhizium* (*robertsii*) and *Beauveria* (*bassiana*). *MycoKeys* 38, 1–23. https://doi.org/10.3897/mycokeys.38.26970.

St. Leger, R. J. (2008). Studies on adaptations of *Metarhizium anisopliae* to life in the soil. *Journal of Invertebrate Pathology,* 98, 271–276. https://doi.org/10.1016/j.jip.2008.01.007.

Togni, P. H. B., Venzon, M., Souza, L. M., Santos, J. P. C. R., & Sujii, E. R. (2019a). Biodiversity provides whitefly biological control based on farm management. *Journal of Pest Science,* 92, 393–403. https://doi.org/10.1007/s10340-018-1021-x.

Togni, P. H. B., Venzon, M., Souza, L. M., Sousa, A. A. T. C., Harterreiten-souza, É. S., Pires, C. S. S., ... Sujii, E. R. (2019b). Dynamics of predatory and herbivorous insects at the farm scale: the role of cropped and noncropped habitats. *Agriculture and Forest Entomology,* 21, 351–362. https://doi.org/10.1111/afe.12337.

Townsend, R., Glare, T., & Willoughby, B. (1995). The fungi *Beauveria* spp. cause epizootics in grass grub populations in Waikato. in *Proceedings of the 48th New Zealand plant protection conference* (New Zealand), 237–241.

Venzon, M. (2021). Agro-ecological management of coffee pests in Brazil. *Frontiers in Sustainable Food Systems*, 5, 721117. https://doi.org/10.3389/fsufs.2021.721117

Venzon, M. ., Togni, P. H. B. ., Chiguachi, J. A. M. ., Pantoja, G. M. ., Brito, E. A. S. ., & Sujii, E. R. (2019). Agrobiodiversidade como estratégia de manejo de pragas. *Informe*

Agropecuário 40, 21–29.

Wekesa, V. W., Knapp, M., & Delalibera, I. (2008). Side-effects of pesticides on the life cycle of the mite pathogenic fungus *Neozygites floridana*. *Experimental and Applied Acarology,* 46, 287–297. https://doi.org/10.1007/s10493-008-9168-4.

Wilson, D. (1995). Endophyte - the evolution of a term, and clarification of its use and definition. *Oikos* 73, 274–276.

Winding, A., Bach, E., & Mele, P. (2020). "Contributions of soil biodiversity to ecosystem functions and services," in *State of Knowledge of soil biodiversity: Status, challenges and potentialities*, eds. FAO, ITPS, GSBI, CBD, and EC (Roma: FAO), 115–190.

Wyrebek, M., Huber, C., Sasan, R. K., & Bidochka, M. J. (2011). Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. *Microbiology* 157, 2904–2911. https://doi.org/10.1099/mic.0.051102-0.

Tables

Table 1. Comparison of the survival of *T. molitor* in roots from diversified and conventional coffee systems. Collection was made in September 2020 (dry season). The statistical values represent the contrast analysis (t test) between plants roots.

Contrast	t ratio	p value
Conventional coffee *versus* Diversified coffee	2.080	0.2918
Conventional coffee *versus Inga edulis*	2.852	0.0130
Conventional coffee *versus Varronia curassavica*	0.7210	1.000
Conventional coffee *versus Senna macranthera*	2.383	0.243
Conventional coffee *versus Gnaphalium spicatum*	0.0266	1.000
Conventional coffee *versus Conyza bonariensis*	0.0741	1.000
Conventional coffee *versus Solanum americano*	15.705	1.000
Diversified coffee *versus Inga edulis*	0.7722	0.6909
Diversified coffee *ver*sus Varronia curassavica	1.359	0.9142
Diversified coffee *versus Senna macranthera*	0.3028	1.000
Diversified coffee *versus Gnaphalium spicatum*	2.826	0.2997
Diversified coffee v*ersus Conyza bonariensis*	2.006	0.8554
Diversified coffee *versus Solanum americano*	17.785	1.000
Inga edulis versus Varronia curassavica	2.131	0.2372
Inga edulis versus Senna macranthera	0.4694	0.999
Inga edulis versus Gnaphalium spicatum	2.826	0.2997
Inga edulis versus Conyza bonariensis	2.778	0.3288
Inga edulis versus Solanum americano	1.855	1.000
Varronia curassavica versus Senna macranthera	1.662	0.8324
Varronia curassavica versus Gnaphalium spicatum	0.6944	1.000
Varronia curassavica versus Conyza bonariensis	0.6469	1.000
Varronia curassavica versus Solanum americano	16.426	1.000
Senna macranthera versus Gnaphalium spicatum	2.356	0.7310
Senna macranthera versus Conyza bonariensis	2.309	0.7599
Senna macranthera versus Solanum americano	18.088	1.000
Gnaphalium spicatum versus Conyza bonariensis	0.0475	1.000
Gnaphalium spicatum versus Solanum americano	15.732	1.000
Conyza bonariensis versus Solanum americano	15.779	1.000

Table 2. Comparison of the survival of *T. molitor* in roots from diversified and conventional coffee systems. Collection was made in January 2021 (rainy season). The statistical values represent the contrast analysis (t test) between plants roots.

Contrast	t ratio	p value
Conventional coffee *versus* Diversified coffee	0.8699	0.688
Conventional coffee *versus Inga edulis*	2.178	<0.001
Conventional coffee *versus Varronia curassavica*	1.451	0.033
Conventional coffee *versus Senna macranthera*	2.211	<0.001
Conventional coffee *versus Ageratum conyzoides*	1.498	0.022
Conventional coffee *versus Bidens Pilosa*	1.365	0.0601
Conventional coffee *versus Sida cordifolia*	0.8177	0.9094
Diversified coffee *versus Inga edulis*	1.308	0.0003
Diversified coffee *versus Varronia curassavica*	0.5816	0.8981
Diversified coffee *versus Senna macranthera*	1.341	0.0113
Diversified coffee *versus Ageratum conyzoides*	0.6288	0.8981
Diversified coffee *versus Bidens pilosa*	0.4958	0.9685
Diversified coffee *versus Sida cordifolia*	0.0523	1.000
Inga edulis versus Varronia curassavica	0.7272	0.5183
Inga edulis versus Senna macranthera	0.0325	1.000
Inga edulis versus Ageratum conyzoides	0.6800	0.5183
Inga edulis versus Bidens pilosa	0.8130	0.2863
Inga edulis versus Sida cordifolia	1.361	0.0114
Varronia curassavica versus Senna macranthera	0.7596	0.7733
Varronia curassavica versus Ageratum conyzoides	0.0472	1.000
Varronia curassavica versus Bidens pilosa	0.0858	1.000
Varronia curassavica versus Sida cordifolia	0.6339	0.9573
Senna macranthera versus Ageratum conyzoides	0.7125	0.8480
Senna macranthera versus Bidens pilosa	0.8455	0.5869
Senna macranthera versus Sida cordifolia	1.393	0.0553
Ageratum conyzoides versus Bidens pilosa	0.1330	1.000
Ageratum conyzoides versus Sida cordifolia	0.6810	0.9234
Bidens pilosa versus Sida cordifolia	0.5480	0.9877

Table 3. Comparison of the number of colony-forming units (CFU) of *Metarhizium* spp. of in roots from diversified and conventional coffee systems. Collection was made in September 2020 (dry season). The statistical values represent the contrast analysis (z test) between plants roots.

Contrast	z ratio	p value
Conventional coffee *versus* Diversified coffee	0.857	0.9897
Conventional coffee *versus Inga edulis*	3.180	0.0318
Conventional coffee *versus Varronia curassavica*	0.863	0.1038
Conventional coffee *versus Senna macranthera*	0.074	1.000
Conventional coffee *versus Gnaphalium spicatum*	0.302	1.000
Conventional coffee *versus Conyza bonariensis*	0.426	0.9999
Conventional coffee *versus Solanum americano*	0.211	1.000
Diversified coffee *versus Inga edulis*	3.104	0.0402
Diversified coffee *ver*sus Varronia curassavica	2.502	0.1943
Diversified coffee *versus Senna macranthera*	0.537	0.9995
Diversified coffee *versus Gnaphalium spicatum*	0.857	0.9896
Diversified coffee v*ersus Conyza bonariensis*	0.308	1.000
Diversified coffee *versus Solanum americano*	0.494	0.9997
Inga edulis versus Varronia curassavica	0.672	0.9977
Inga edulis versus Senna macranthera	1.638	0.7269
Inga edulis versus Gnaphalium spicatum	2.171	0.3695
Inga edulis versus Conyza bonariensis	2.375	0.2536
Inga edulis versus Solanum americano	2.364	0.2593
Varronia curassavica versus Senna macranthera	1.456	0.8306
Varronia curassavica versus Gnaphalium spicatum	1.952	0.5151
Varronia curassavica versus Conyza bonariensis	2.012	0.4737
Varronia curassavica versus Solanum americano	2.040	0.4543
Senna macranthera versus Gnaphalium spicatum	0.160	1.000
Senna macranthera versus Conyza bonariensis	0.336	1.000
Senna macranthera versus Solanum americano	0.206	1.000
Gnaphalium spicatum versus Conyza bonariensis	0.594	0.999
Gnaphalium spicatum versus Solanum americano	0.438	0.999
Conyza bonariensis versus Solanum americano	0.180	1.000

Table 4. Comparison of the number of colony-forming units (CFU) of *Metarhizium* spp. of in roots from diversified and conventional coffee systems. Collection was made in January 2021 (rainy season). The statistical values represent the contrast analysis (z test) between plants roots.

Contrast	z ratio	p value
Conventional coffee *versus* Diversified coffee	3.410	0.0150
Conventional coffee *versus Inga edulis*	3.694	0.0054
Conventional coffee *versus Varronia curassavica*	2.666	0.1331
Conventional coffee *versus Senna macranthera*	2.819	0.0904
Conventional coffee *versus Ageratum conyzoides*	2.627	0.1460
Conventional coffee *versus Bidens pilosa*	2.608	0.1530
Conventional coffee *versus Sida cordifolia*	4.400	0.0003
Diversified coffee *versus Inga edulis*	0.885	0.0003
Diversified coffee *versus Varronia curassavica*	1.165	0.9417
Diversified coffee *versus Senna macranthera*	0.469	0.9998
Diversified coffee *versus Ageratum conyzoides*	1.222	0.9257
Diversified coffee *versus Bidens pilosa*	1.250	0.9167
Diversified coffee *versus Sida cordifolia*	2.929	0.0669
Inga edulis versus Varronia curassavica	1.746	0.6566
Inga edulis versus Senna macranthera	0.999	0.9747
Inga edulis versus Ageratum conyzoides	1.795	0.6237
Inga edulis versus Bidens pilosa	1.819	0.6070
Inga edulis versus Sida cordifolia	1.695	0.6908
Varronia curassavica versus Senna macranthera	0.432	0.9999
Varronia curassavica versus Ageratum conyzoides	0.056	1.000
Varronia curassavica versus Bidens pilosa	0.084	1.000
Varronia curassavica versus Sida cordifolia	3.173	0.0325
Senna macranthera versus Ageratum conyzoides	0.479	0.9997
Senna macranthera versus Bidens pilosa	0.503	0.9996
Senna macranthera versus Sida cordifolia	2.133	0.3934
Ageratum conyzoides versus Bidens pilosa	0.028	1.000
Ageratum conyzoides versus Sida cordifolia	3.208	0.0291
Bidens pilosa versus Sida cordifolia	3.226	0.0276

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Figures

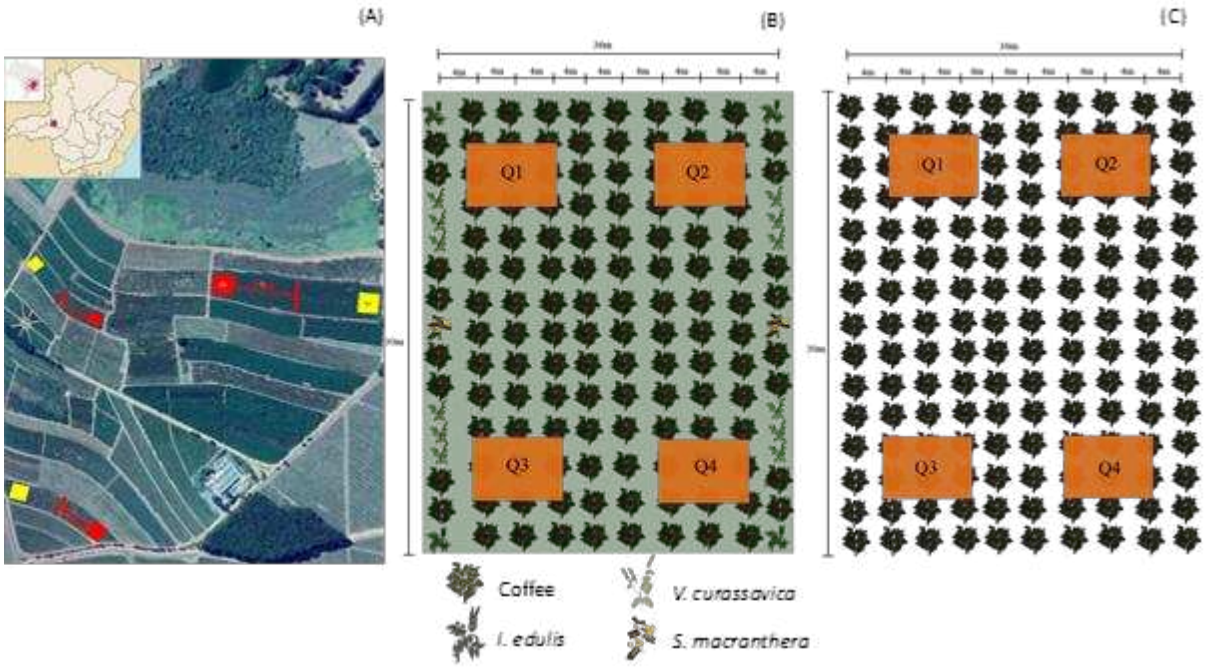

Figure 1. Sketch of the experimental design. (A) Location of plots in the Experimental Research Station of EPAMIG Patrocínio, Cerrado of Minas Gerais, Brazil. Red squares represented plots with diversified coffee system and yellow squares plots with conventional coffee system. (B) Plot with diversified coffee system. (C) plot with conventional coffee system. Each plot measured 1080 m². Brown square delimit four sampling quadrants.

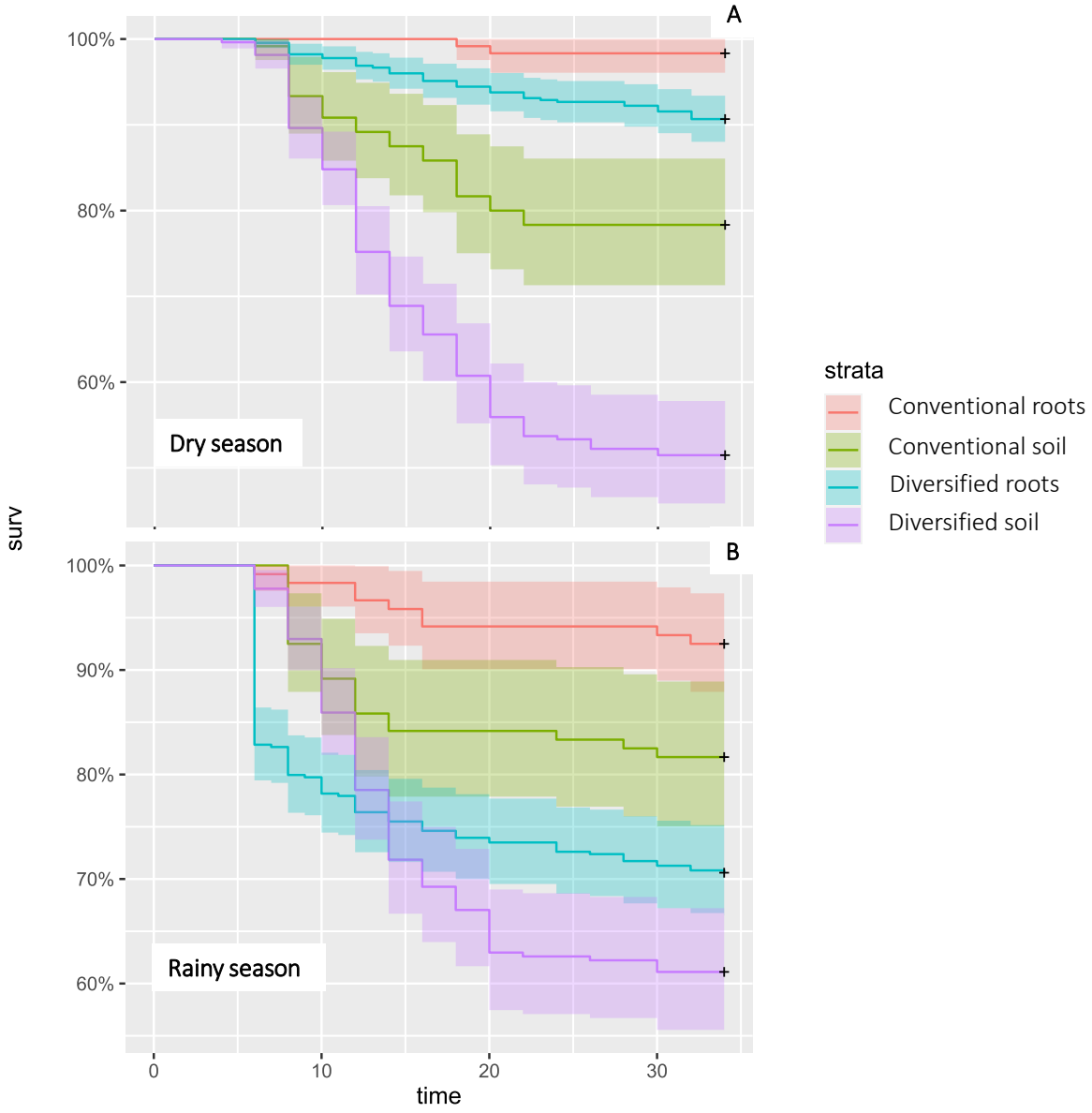

Figure 2. Survivorship of *T. molitor* bait insect larvae in soil and root plants from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. A: Collect in September 2020 – dry season. B: Collect in January 2021 – rainy season. Mortality of bait insects was evaluated for 35 days. Analyses are presented in the text.

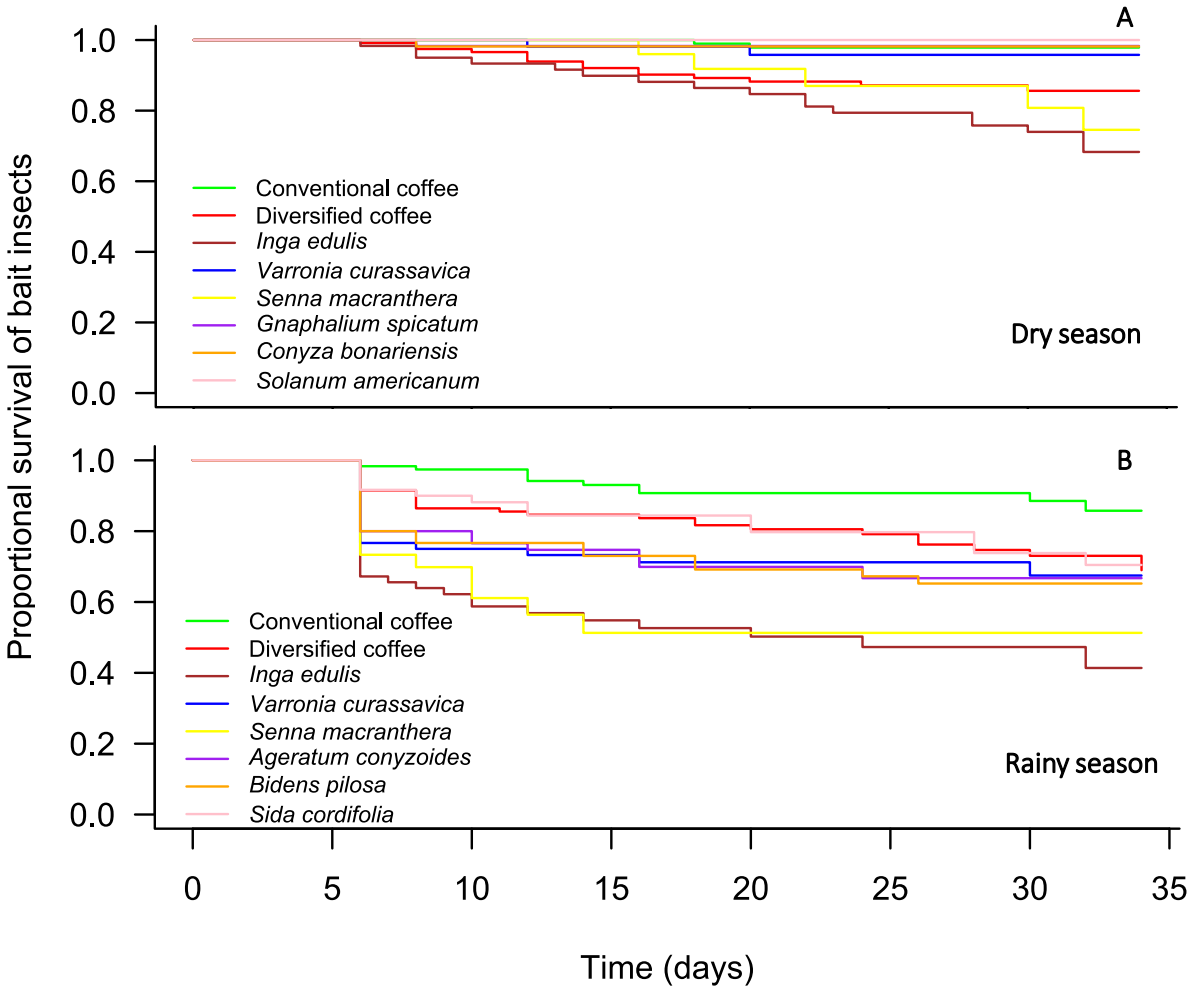

Figure 3. Survivorship of *T. molitor* bait insect larvae in root plants from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. A: Collect in September 2020 – dry season. B: Collect in January 2021 – rainy season. Mortality of bait insects was evaluated for 35 days. Analyses are presented in the text.

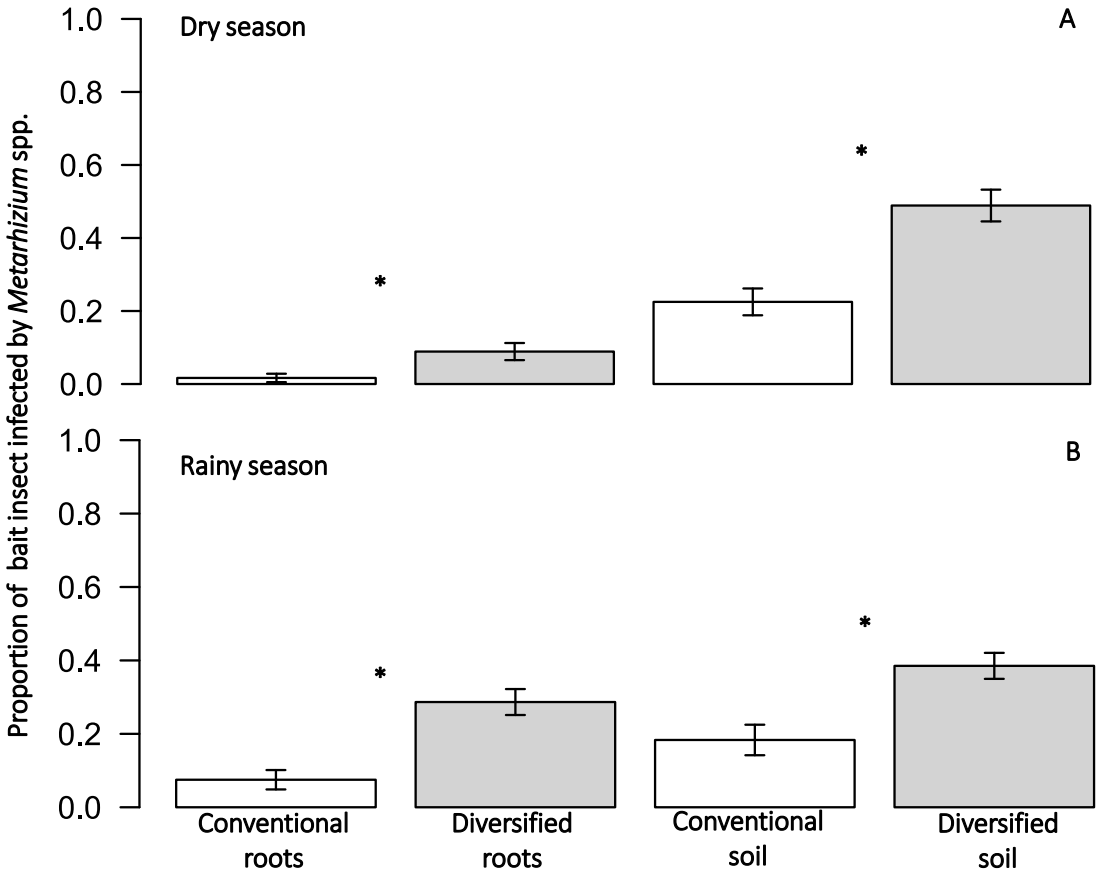

Figure 4. Proportion of bait insect *T. molitor* infected by *Metarhizium* spp. in soil and roots from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. A: Collect in September 2020 – dry season. B: Collect in January 2021 – rainy season. Analyses are presented in the text. *$p<0.05$, ns - not significant.

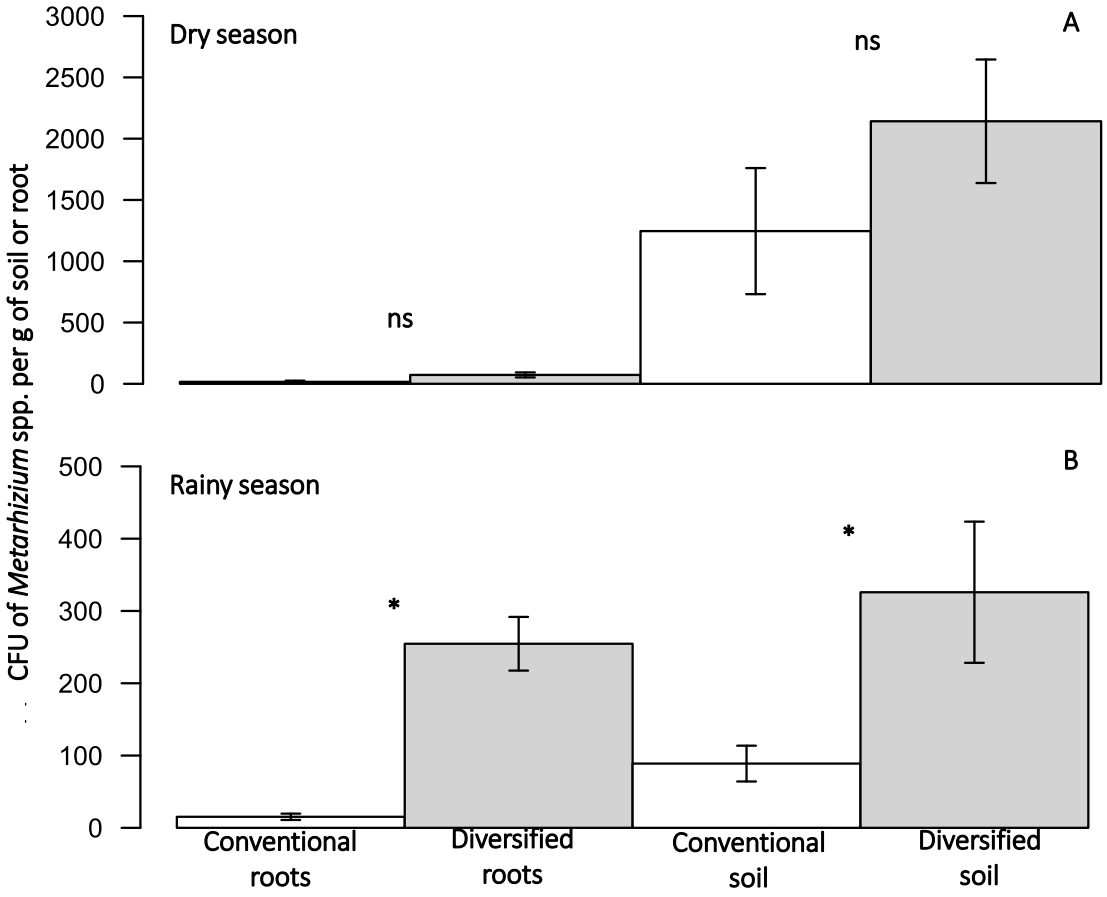

Figure 5. Number of colony-forming units (CFU) (mean ±standard error) of *Metarhizium* spp. in soil and roots from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. A: Collect in September 2020 – dry season. B: Collect in January 2021 – rainy season. Analyses are presented in the text. *$p<0.05$, ns - not significant.

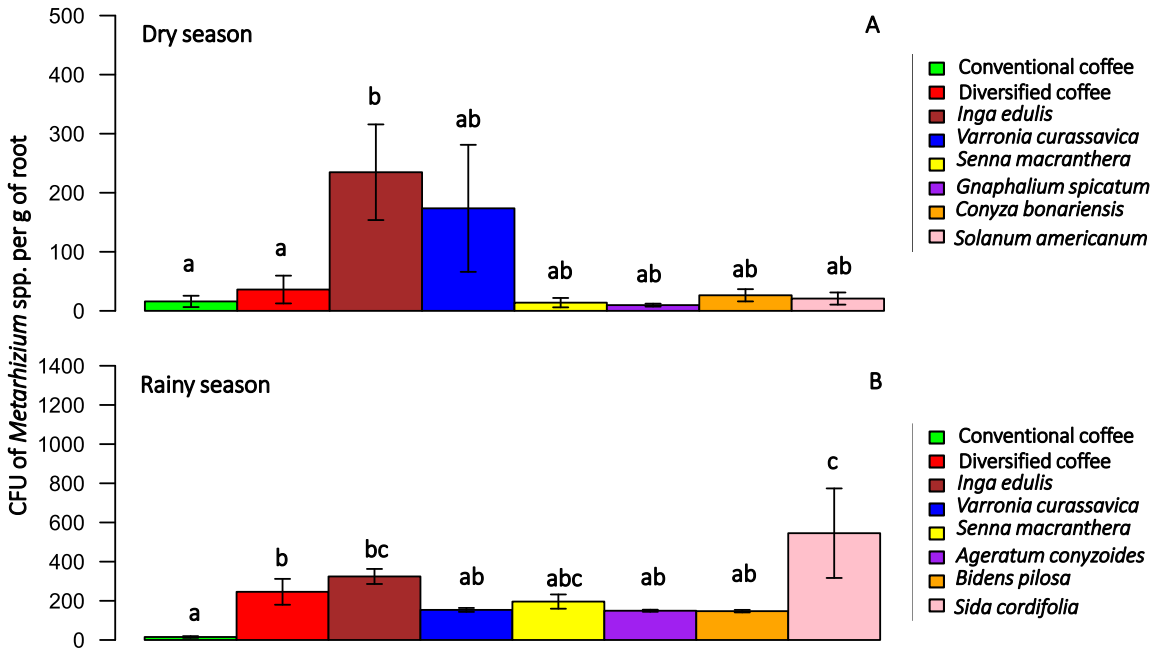

Figure 6. Number of colony-forming units (CFU) (mean ± standard error) of *Metarhizium* spp. in plant roots from conventional and diversified coffee systems in Patrocínio, Cerrado of Minas Gerais, Brazil. A: Collect in September 2020 – dry season. B: Collect in January 2021 – rainy season. Analyses are presented in the text. Bars with same letter have no significant differences.

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Chapter III[1]

Coffee seedlings treatment by *Metarhizium*: plant growth and protection against *Leucoptera coffeella*

ABSTRACT

Metarhizium fungi are entomopathogenics and plant mutualists. They are able to colonize plant roots and promote plant growth and protection against pests. We found *Metarhizium robertsii* and *M. brunneum* associated with coffee roots from diversified coffee system in Cerrado of Minas Gerais. Here, we investigated whether coffee seedlings inoculated by soil drench with these species of *Metarhizium* can associate with coffee roots and improve coffee seedlings growth and indirectly protect against coffee leaf miner (CLM) *Leucoptera coffeella* (Lepidoptera: Lyonetiidae), which is a pest that damage coffee plants since the nursery period. We performed a greenhouse experiment with coffee seedlings using 30 ml of suspension (10^8) of each *Metarhizium* species as applied as soil drench to each potted seedling. Control seedlings received a soil drench of sterile solution of Tween 0.05 %. After eight days of inoculation, we infested the seedlings with two couples of CLM adults and evaluated colonization of *Metarhizium*, CLM development and seedling growth. We recovered *Metarhizium* spp. from most of coffee seedlings roots. Both isolates promoted protection against CLM in coffee seedlings, reducing the percentage of mined leaf area and prolonging CLM development time, compared to control. Besides, coffee seedlings inoculated with *M. robertsii* had greater leaf area than seedlings with *M. brunneum* and control. Our results showed that *M. brunneum* and *M. robertsii* associated with coffee seedling roots promoting protection against CLM and *M. robertsii* also improve coffee seedling growth. Therefore, these *Metarhizium* species could be considered for the development of inoculants for coffee seedlings.

Keywords: Endophytes, coffee leaf miner, *Metarhizium robertsii*, *Metarhizium brunneum*.

[1] Chapter formatted in the norms of the Biological Control

1 Introduction

The fungal genera *Metarhizium* (Hypocreales: Clavicipitaceae) can infect arthropods (Brunner-Mendoza et al., 2019; David, 1967; Jaramillo et al., 2015; Meyling and Eilenberg, 2007) and endophytically colonize plants (Behie et al., 2012; Behie and Bidochka, 2014; Fisher et al., 2011; Keyser et al., 2015; Krell et al., 2018). *Metarhizium* species cause fungal diseases in economically important arthropod pests, such as termites, locusts, grasshoppers, cockroaches, white flies, thrips, mosquitoes and ticks (Zimmermann, 2007; Roberts and Hajek, 1992), which made them are important tools to biological control (Brunner-Mendoza et al., 2019; Kepler et al., 2015). The use of *Metarhizium* as biological agent is made mainly as an inundative control (Roberts and Hajek, 1992) and there are several commercial products based on *Metarhizium* registered around the world (Zimmermann, 2007).

Metarhizium species in association with the host plant can confer benefits as growth promotion (Ahmad et al., 2020; Jaber and Araj, 2018; Liao et al., 2014), nutrient transfer (Behie et al., 2012) and protection against insect pests (Ahmad et al., 2020; Jaber and Araj, 2018) and diseases (Chairin and Petcharat, 2017; Sasan and Bidochka, 2013). In turn, the host plant provides fungi with photosynthetic compounds (Behie et al., 2017). This association can be endophytic (Behie et al., 2012) or rhizosphere competence (Hu and Leger, 2002). Endophytic fungi develop within plant tissues without causing any noticeable symptoms of disease in the plant (Mantzoukas and Eliopoulos, 2020; Wilson, 1995). On other hand, rhizosphere-competence fungi grow in the rhizosphere without colonize plant tissues (Morgan et al., 2005). *Metarhizium* species associate with root (Ahmad et al., 2020; Behie et al., 2015), stem and leaf (Ahmad et al., 2020; Batta, 2013). However, the most common is inhabit root (Ahmad et al., 2020; Bamisile et al., 2018; Wyrebek et al., 2011).

The ability of *Metarhizium* species to associate with plants is also corroborated by genomic studies. Gao and collaborators showed in a phylogenomic study that *Metarhizium*

genus is more closely related to the grass endophytes, *Claviceps* and *Epichloë* than to animal pathogens (Gao et al., 2011). They also showed that the genus harbors genes that codify plant degrading enzymes in its genomes, which is indicative that *Metarhizium* genus may have evolved from a plant symbiont lineage, as it was also proposed by Spatafora et al. (2007). Some species in the genus have not left the role as plant symbionts, and subsequently acquired the ability to infect and kill insects (Barelli et al., 2016; Hu et al., 2014).

Colonization of plant roots by fungi increases the surface area from which plants can scavenge nutrients, facilitating the absorption of soil nutrients, which results in increased photosynthetic ability and enhanced plant growth (Clark and Zeto, 2000). The protection against herbivores could be due production of fungal secondary metabolites *in plant. Metarhizium* can produce destruxins (Roberts, 1981), a secondary metabolite toxic to insects that was detected in cowpea plants (Golo et al., 2014), potato (Ríos-Moreno et al., 2016) and tomato leaves (Resquín-Romero et al., 2016). These responses *in plant* can be play by abscisic acid (ABA) which reduces immune responses during endophytic colonization by *Metarhizium robertsii* and induces immune responses to pathogenic colonization by *Fusarium solani,* during the early stages of fungal infection in bean plants (Hu and Bidochka, 2021). However, some studies appointee that protection can be caused by the induction of plant resistance, since the fungi colonization can at first be recognized as potential invaders triggering immune responses such as transcription factors involved in the resistance against herbivores (Brotman et al., 2013; McKinnon et al., 2017).

Several studies have reported the indirect negative effects of *Metarhizium* species on herbivores feeding in above ground plant parts, however most of the then did not described the mechanism that is triggering these effects. Canassa et al. (2019) found that root colonization of *Phaseolus vulgaris* by *M. robertsii* can suppress spider mites feeding on above ground and improve plant growth. *Metarhizium. brunneum* inoculated on the roots of sweet pepper

Capsicum annum increased plant growth and prolonged development time, delayed onset of reproduction, and reduced birth rate of *Myzus persicae* (Jaber and Araj, 2018). Maize seeds treated with *M. brunneum*, *M. anisopliae* and *M. robertsii* increase leaf collar formation, stalk length, average ear biomass and average stalk and foliage biomass (Liao et al., 2014).

The coffee leaf miner (CLM) *Leucoptera coffeella* (Lepidoptera: Lyonetiidae) is a pest that damage coffee plants since the nursery, which reduces the seedlings quality through the reduction of leaf area and defoliation (Reis et al. 2002, Tomaz et al., 2015). This pest often attains high population levels in many coffee production regions in Brazil, which may cause defoliation up to 70%, reducing coffee yields by 50% (Reis and Souza, 1996). Thus, it is important to produce healthy coffee seedlings in order to avoid planting CLM infested seedlings. The use of microbes as inoculant to promote plant health may protects coffee seedlings against CLM, consequently, avoid high CLM infestations in coffee crops. Therefore, studies about a potential coffee seedling inoculant can provide a control strategy for this pest.

During our field studies, we isolated *M. robertsii* and *M. brunneum*, by bait insect method (Zimmermann, 1986), from coffee roots collected in a diversified coffee system in the Patrocínio in Cerrado of Minas Gerais. Here, we evaluated the two *Metarhizium* species inoculated in coffee seedlings roots by soil drench and hypothesize that (a) *Metarhizium* spp. indirectly promotes protection against CLM by colonization of coffee roots and (b) improves the seedlings growth.

2 Material and Methods

2.1 Fungal isolates, plants and insects

We used the entomopathogenic fungi (EPF) isolates RD-20.114 of *M. robertsii* and RD-20.120 of *M. brunneum,* kept in tube with slant PDA at 5 ºC. We obtained these two isolates from coffee roots, using the *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae as a bait insect

(Fisher et al., 2011; Zimmermann, 1986), collected in a diversified coffee system at Experimental Research Station of Agriculture and Livestock Research Enterprise of Minas Gerais (EPAMIG) in Patrocínio, Cerrado region of Minas Gerais.

The coffee seedlings - variety "IAC 44" - were obtained from the coffee nursery of the Plant Pathology Department of the Federal University of Viçosa (UFV) - Viçosa, MG, Brazil. They were three month old after the sowing the seed ("orelha de onça" stage: the first two round-shaped leaves). These seedlings were cultivated without pesticides application and were fully inspected to ensure the absence of pests and diseases before setting up the experiment. The coffee seedlings were transplanted in 3 l pots containing substrate (MecPlant®) and kept in a greenhouse until fungal inoculation. Seedling fertilization was done monthly containing with 4g l^{-1} of ammonium sulfate. The irrigation was done with 30 ml of water per seedling every two days. The experiment was set up three months after the transplantation, when plants had at least six pairs of leaves.

We collected CLM infested leaves from plants at Diogo Alves de Mello Experimental Station at UFV - Viçosa, MG, Brazil. The rearing was kept in the Laboratory of Agroecology of EPAMIG - Viçosa, MG, Brazil, at 23±1 ºC and 12:12 (L:D). Mined leaves were maintained in transparent acrylic cages (40 ×40 × 40 cm) with their petioles inserted into plastic boxes (20 × 10 cm) with foam sections soaked in water. When adults emerged from infested leaves, we transferred them to new cages with clean coffee leaves to allow the continuity of CLM life cycle (Martins et al., 2021).

2.2 Fungal suspensions

Metarhizium robertsii and *M. brunneum* from stock cultures were plated on Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) supplemented with 0.05 g l^{-1} chloramphenicol and incubated in darkness at 26 °C for 15 days. We autoclaved bags with 100

g of rice (polished parboiled type 1) with 30 ml of sterile distilled water for 15 minutes at 1.0 atm pressure to 120 ºC. After that, the bags were kept inside of laminar flow until the complete cooling. Subsequently, *Metarhizium* conidia were harvested from the Petri dishes and inoculated into the plastic bags containing rice. Rice bags were kept t in darkness at 26 °C for 7 days. After that, rice grains with conidia were suspended in sterile Tween solution 0.05%. Subsequently, the suspensions were filtrated twice using a two layers sterile cheesecloth for removing rice grains and hyphal fragments and vortexed for 30 seconds.

The concentration of suspensions was adjusted to 1×10^8 conidia ml^{-1} in sterile tween solution 0.05%. We checked conidial viability by transferring 150 µl of the suspension onto Petri dishes (9 cm diameter) with PDA + chloramphenicol (0.05 g l^{-1}) and counted germinated conidia after 24 h, at 26 °C. Suspension of both isolates presented germination rates higher than 98%.

2.3 Treatments

We inoculated, by soil drench, 30 ml of the suspension of each isolate at the soil close to the coffee seedling stem. Controls received 30 ml of sterile solution of Tween 0.05%. The three treatments were *M. robertsii, M. brunneum* and control with 20 coffee seedlings for each treatment. Each potted coffee seedling was kept in a cylindric cage (30 cm diameter x 60 cm height) made with wire rod (3 mm diameter) and covered with voile (Figure 1). The experiment was maintained into a greenhouse at the Laboratory of Insect-Microorganism Interactions, UFV.

2.4 Effects of *M. robertsii* and *M. brunneum* on CLM

Eight days after coffee seedling had received the treatments, we infested each seedling with two couples of CLM. After 48 h, we removed the adults and counted the eggs laid by the

females on each seedling with a pocket magnifier. From this time, we daily evaluated CLM development time and the number of mines, pupae and adults on each seedling. The seedlings were evaluated until there was no more adult emergence, about to 40 days. In the last evaluation day, we removed all leaves from seedling and took pictures of them. We evaluated the percentage of mined leaf area, measuring the total leaf area and mined area of the pictures with the ImageJ software (Abramoff et al., 2004).

We evaluated the survival and reproductive performance of CLM adults emerged from seedlings. For this, we used 26 CLM couples formed from adults emerged from *M. robertsii*, 30 from *M. brunneum* and 30 from control. Each couple was placed an inside plastic pot (500 ml) containing one clean and untreated coffee leaf from coffee seedlings maintained in greenhouse, covered with PVC plastic. The leaf petiole was inserted in a plastic container (3 ml) with water to maintain leaf turgidity (Martins et al., 2021). We evaluated the survival of males and females and number of eggs per female under microscope daily until their death.

2.5 Effects of *M. robertsii* and *M. brunneum* on coffee seedling growth

To evaluate whether *Metarhizium* species have the potential to promote the growth of coffee seedlings, we evaluated the number of leaves, leaf area, length of aerial part and root, stem diameter, and fresh and dry weight of aerial part and roots. Since we used seedlings with different initial sizes, we measured the length of aerial part and the number of leaves of each coffee seedling prior to treatments. At the end of the experiment, 43 days after fungal inoculation, we repeated aerial part measurement and leaf counting. Then, we subtract the initial values from final values and evaluated the gain during the experiment. We used the ImageJ software to measure leaf area, tape measure to evaluate the length, digital caliper to measure the diameters and a precision balance to evaluate the weight.

2.6 Evaluation of the colonization of *M. robertsii* and *M. brunneum* in coffee seedlings roots

We collected roots from inoculated and non-inoculated coffee seedlings at the end of the experiment, in order to evaluate the presence or absence of *Metarhizium*. We shake each root in a standardized manner to remove non-rhizosphere soil. Subsequently, we ground 2 g of roots of each plant individually with mortar and pestle and then suspended them in 5 ml of sterile Tween solution at 0.01%, in a Falcon® tube (15 ml). Tubes were rotated for one hour in a rotary shaker at 150 rpm (Lacey, 2012). The suspensions were then vortexed for 15 seconds and an aliquot of a 100 µl from each suspension were plated in three Petri dishes (9 cm diameter) with selective medium, and spread with a sterile Drigalski spatula. We used a selective medium with 10 g peptone, 20 g dextrose, 15 g agar, and 1 L distilled water. After sterilizing the medium, we added 0.05 g l^{-1} of cycloheximide and tetracycline, 0.6 g l^{-1} streptomycin and 0.175 g l^{-1} CTAB (cetyltrimethylammonium bromide) (Kepler et al., 2015). Petri dishes were incubated in darkness at 26 °C for 14 days. After the incubation period, we evaluated the frequency of fungal colonization in roots. From the inoculated plants, only the plant that present at least one *Metarhizium* colony were considered positive for fungal association.

2.8 Statistical analyses

We used inoculation, *M. robertsii*, *M. brunneum* and control, as explanatory variables to investigate the effect of *Metarhizium* species on CLM parameters and coffee seedling growth. Survival analyses with censored Weibull distributions was carried out to test CLM development time. We used generalized linear mixed models (GLM) adjusted to Poisson distribution to analyze CLM count data of number of eggs, mines, pupae and emerged adults per plant. Analyses were ANOVA with χ^2 tests and pairwise comparisons were performed by emmeans (Lenth et al., 2021). For percentage of mined leaf area, we used Analysis of Deviance and pairwise comparisons were performed by emmeans. From emerged CLM adults, we examined

the survival of males and females by survival analyses with exponential distribution. For numbers of eggs per emerged CLM female, we used GLM adjusted to Poisson distribution and analyzed by ANOVA with χ^2 tests. To examine coffee seedling growth variables (number of leaves, leaf area, stem diameter, length of roots and aerial part, fresh and dry mass of roots and aerial part) we used Analysis of Deviance test and pairwise comparisons were performed by emmeans. To examine the frequencies with which the fungi were re-isolated in coffee seedling roots, they were scored as positive or negative for *M. robertsii* or *M. brunneum*. Based on the confirmation of the colonization by *Metarhizium* species we excluded the inoculated coffee seedlings that was negative for fungus from the analyses. We used R (R Core Team, 2018) to analyze all data.

3 Results

3.1 Effects of *M. robertsii* and *M. brunneum* on CLM

In coffee seedlings inoculated with *M. robertsii* (z=2.39, p=0.04) and *M. brunneum* (z=5.68, p<0.001) CLM presented delayed of two days on developmental time, compared to control treatment (Figure 2). There was no difference on CML development time between *M. robertsii* and *M. brunneum* (z=1.95, p=0.12, Figure 2). However, the inoculation of both fungi did not decrease the numbers of eggs (χ^2=1.95, p=0.88, df=2, Figure 3A), mines (χ^2=24.43, p=0.11, df=2, Figure 3B) and pupae (χ^2=26.59, p=0.11, df=2, Figure 3C). Nevertheless, the inoculation of *M. robertsii* reduced the numbers of CLM emerged adults by a third, compared to control treatment (z=2.86, p=0.01, Figure 3D). Additionally, the percentage of mined leaf area was lower in *M. robertsii* (0.08±0.02 cm^2, t=7.68, p<0.001) and *M. brunneum* (0.28±0.10 cm^2, t=6.11, p<0.001) treatments, compared to control treatment (1.38±0.17 cm^2) (Figure 4). There was no difference of mined leaf area between *M. robertsii* and *M. brunneum* (t= 1.09, p=0.52, Figure 4).

The survival of emerged females (χ^2=3.37, p=0.18, df=2) and males (χ^2=2.82, p=0.24, df=2) were similar in all treatments. The number of eggs per emerged female was two times lower in *M. robertsii* treatment compared to control treatment (z=2.46, p=0.03, Figure 5). However, there was no difference between *M. brunneum* and control treatment (z=1.89, p=0.13, Figure 5) and *M. robertsii* (z=0.77, p=0.71, Figure 5)

3.2 Effects of *M. robertsii* and *M. brunneum* on coffee seedlings growth

The number of leaves (F=1.55, p=0.22, df=2; Figure 6A), length of aerial part (F=0.37, p=0.68, df=2; Figure 6B) and roots (F=0.29, p=0.743, df=2; Figure 6C), stem diameter (F=1.23, p=0.30, df=2; Figure 6D), fresh mass of roots (F=0.19, p=0.82, df=2; Figure 6E) and aerial part (F=0.29, p=0.74, df=2; Figure 6F) and dry mass of roots (F=0.78, p=0.46, df=2; Figure 6G) and aerial part (F=0.37, p=0.69, df=2; Figure 6H) were similar in all treatments. However, the inoculation of *M. robertsii* in coffee seedlings increased 30% the leaf area compared to control treatment (t= 6.253, p<0.001; Figure 6I).

3.3 Colonization of *M. robertsii* and *M. brunneum* in coffee seedlings roots

Both isolates were recovered from fungus treated coffee seedlings roots. At 43 days after the inoculations, *M. robertsii* was recovered in 90% of coffee seedlings roots and *M. brunneum* from 75% of coffee seedlings roots (Figure 7). Besides, no *Metarhizium* isolate was recovered from coffee seedlings roots of control treatment (Figure 7).

4 Discussion

Both isolates promoted some level of protection against CLM in coffee seedlings, reducing percentage of mined leaf area and prolonging development time, supporting our

hypothesis. Besides, *M. robertsii* inoculation reduced the numbers of adults and the number of eggs per females, that emerged from inoculated plants. Endophytic EPF may promote protection against pests, increasing rates of infection and mortality of insect pests (Ahmad et al., 2020; Canassa et al., 2019; Jaber and Araj, 2018). It has been suggested that the mechanisms of these systemic responses are that fungal metabolites could be excreted and transported through plant vascular system, either directly affecting herbivores or mediating indirect effects through the upregulation of plant defenses (Jaber and Ownley, 2018). The fungal metabolites could be alkaloids, saponins, tannins, phenolic acids, steroids, quinones and terpenoids, that serve as storehouse of unique bioactive secondary metabolites and as insect antagonist (Gouda et al., 2016). Besides, endophytes possibly induce changes in volatile emissions, what can influence the host selection of insect for oviposition (Jallow et al., 2008).

Insect feeding mode is an important aspect to consider in indirectly protect against pest by endophytic EPF, because chewing, sucking, mining and galling insects often respond differently to plant defenses (Gange et al., 2019). Sucking insects are more strongly affected to endophytic EPF inoculation than chewing, mining and galling insects (Gange et al., 2019), because fungal metabolites could be excreted and transported through plant vasculature (Jaber and Ownley, 2018). It is argued that intracellular entomopathogenic fungal growth is limited (Ullrich et al., 2017). Here, we investigated the indirect protection by *Metarhizium* spp. against a mining insect and we found negatives effects when it was in mining stage, decreasing percentage of mined leaf area. Therefore, our results suggested that *Metarhizium* inoculation can affect mining insect even though intercellular growth is thought to be limited (Ullrich et al., 2017).

The mutualistic association of *Metarhizium* with plant hosts was reposted as endophytic or rhizosphere competent. An endophytic fungus can develop within plant tissues without causing any noticeable symptoms of disease (Mantzoukas and Eliopoulos, 2020; Wilson, 1995).

On other hand, rhizosphere-competent fungi grow preferentially in the rhizosphere without colonizing plant internal tissues (Morgan et al., 2005). Because we did not sterilize the surface of coffee seedling roots, it was not possible to prove that *Metarhizium* spp. colonized them as endophytic or growing on the root surface. Despite this, we recovered *Metarhizium* spp. without root surface sterilization due to porous root tissues (Taiz et al., 2017), which sterilizing liquids can penetrate and eliminate all of the viable endophytic propagules. Barelli et al. (2018) reported a failure to recover *Metarhizium* from surface sterilized bean roots. The authors believed that it was due to excessive time or concentration of sodium hypochlorite used as sterilizing. Therefore, it is necessary molecular techniques that detect DNA from the entomopathogenic fungal endophyte directly from plant tissue (McKinnon, 2016), however, it become more expensive the detection of endophytic relation. So far, our aim was to show that *Metarhizium* spp. colonize coffee roots, regardless whether it is endophytic or rhizosphere-competence, as initial stages of *Metarhizium* endophytic colonization involves rhizoplane colonization (Barelli et al., 2018). Likewise, in both association, fungi can promote plant growth and protection against pests (Behie et al., 2012; Behie and Bidochka, 2014; Jaber and Araj, 2018) and the EPF fungal/plant associations is transient, as these fungi cannot growth systematically in plant tissues (Barelli et al., 2018).

The inoculation with *M. robertsii* increased the total leaf area of coffee seedlings, indicating that this fungal act as growth promoter in coffee seedlings. EPF colonization may increase plant growth by facilitating nutrient uptake through plant root system or translocate nitrogen from soil insect cadavers to plant in exchange for carbon. (Bamisile et al., 2018; Behie et al., 2012). During our literature review, we did not find any study about *Metarhizium* spp. inoculation in coffee plants. However, studies with other crops have shown that *M. brunneum* inoculation also improves growth plants (Jaber and Araj, 2018; Liao et al., 2014). Although we recovered *M. brunneum* in coffee roots, the isolate did not improve any growth plant variable,

thus, our second hypothesis was not completely supported. The ability of *Metarhizium* to colonize plant tissues varies by fungal species and strain, environmental conditions and host species (Lovett and St. Leger, 2015). *Metarhizium robertsii* occurs region of both earth hemispheres and it is most common found associated to soil and rhizosphere (Sasan and Bidochka, 2012). On the other hand, *M. brunneum* is more restrict on north hemisphere, grow better at low temperatures and shows reduced growth at high temperatures and sensitivity to high UV radiation (Bidochka and Small, 2005). This species is most abundant in soil and rhizosphere of temperate areas (Bidochka and Small, 2005). This could partially explain why *M. robertsii* promotes more growth and protection effects than *M. brunneum* in our experiments.

The development of microbial inoculants that improve coffee seedling growth and have negative effects on CLM is a promissory strategy for reducing of costs with fertilizers and pesticides. More than, it represents a safe strategy to the environmental and human health, which are financially immeasurable. Our results are consistent with studies that report the indirect effect of EPF on reducing insect herbivore damage. The most common effects reported are the delay in the insect developmental time, feeding deterrence, retardation of insect growth, reduction survival and oviposition (reviewed in Bamisile et al., 2018). Studies about inoculation of entomopathogenic fungi in coffee are restricted to *Beauveria bassiana* (Posada et al., 2007; Posada and Vega, 2006), a biological control agent of coffee berry borer *Hypothenemus hampei* Ferrari (Coleoptera: Curculionidae), another key coffee pest worldwide (Oliveira et al., 2013). Our study is novel about *Metarhizium* spp. and their colonization in coffee roots by soil drench, which improves growth plant and protection against CLM. Therefore, our results suggesting *M. brunneum* and *M. robertsii* could be considered for the development of inoculants for coffee seedlings. Further studies are necessary to test the viability of using such strategy in adult coffee plants in the field.

5 Acknowledgments

This work was supported by ‘Conselho Nacional de Desenvolvimento Científico e Tecnológico’ (CNPq), ‘Fundação de Amparo a Pesquisa de Minas Gerais’ (FAPEMIG), 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil' (CAPES) - Finance Code 001, Consórcio Brasileiro de Pesquisa e Desenvolvimento do Café (CBP&D-Café) and Instituto Tecnológico da Vale. We are grateful members of the laboratory of Insect-Microorganism Interactions, UFV, for help with laboratory protocols and the access of the greenhouse. We thank Marcela Caixeta, Jéssica Martins and Verônica Fialho for help to prepared and send *Metarhizium* spp. isolates for molecular characterization. We also thank Jéssica Martins for her support in preparing the cage draft.

6 References

Abramoff, M.D., Magalhães, P. J., Ram, S. J., 2004. Image processing with ImageJ. Biophotonics International, 11, 36 – 42.

Ahmad, I., Jiménez-Gasco, M.M., Luthe, D.S., Shakeel, S.N., Barbercheck, M.E., 2020. Endophytic *Metarhizium robertsii* promotes maize growth, suppresses insect growth, and alters plant defense gene expression. Biol. Control 144, 104167. https://doi.org/10.1016/j.biocontrol.2019.104167

Bamisile, B.S., Dash, C.K., Akutse, K.S., Keppanan, R., Wang, L., 2018. Fungal endophytes: Beyond herbivore management. Front. Microbiol. 9, 544. https://doi.org/10.3389/fmicb.2018.00544

Barelli, L., Moonjely, S., Behie, S.W., Bidochka, M.J., 2016. Fungi with multifunctional lifestyles: endophytic insect pathogenic fungi. Plant Mol. Biol. 90, 657–664. https://doi.org/10.1007/s11103-015-0413-z

Barelli, L., Moreira, C.C., Bidochka, M.J., 2018. Initial stages of endophytic colonization by

Metarhizium involves rhizoplane colonization. Microbiology 164, 1531–1540. https://doi.org/10.1099/mic.0.000729

Batta, Y.A., 2013. Efficacy of endophytic and applied *Metarhizium anisopliae* (Metch.) Sorokin (Ascomycota : Hypocreales) against larvae of *Plutella xylostella* L. (Yponomeutidae : Lepidoptera) infesting *Brassica napus* plants. Crop Prot. 44, 128–134. https://doi.org/10.1016/j.cropro.2012.11.001

Behie, S.W., Bidochka, M.J., 2014. Nutrient transfer in plant–fungal symbioses. Trends Plant Sci. 19, 734–740. https://doi.org/10.1016/j.tplants.2014.06.007

Behie, S.W., Moreira, C.C., Sementchoukova, I., Barelli, L., Zelisko, P.M., Bidochka, M.J., 2017. Carbon translocation from a plant to an insect-pathogenic endophytic fungus. Nat. Commun. 8, 14245. https://doi.org/10.1038/ncomms14245

Behie, S.W., Zelisko, P.M., Bidochka, M.J., 2012. Endophytic insect-parasitic fungi translocate nitrogen directly from insects to plants. Science. 336, 1576–1577. https://doi.org/10.1126/science.1222289

Bidochka, M.J., Small, C.L., 2005. Phylogeography of *Metarhizium*, an insect pathogenic fungus, in: Vega, F.E., Blackwell, M. (Eds.), Insect – Fungal Associations: Ecology and Evolution. Oxford University Press, New York, pp. 28–50.

Brotman, Y., Landau, U., Cuadros-Inostroza, Á., Takayuki, T., Fernie, A.R., Chet, I., Viterbo, A., Willmitzer, L., 2013. *Trichoderma*-plant root colonization: escaping early plant defense responses and activation of the antioxidant machinery for saline stress tolerance. PLoS Pathog. 9, e1003221. https://doi.org/10.1371/journal.ppat.1003221

Brunner-Mendoza, C., Reyes-Montes, M. del R., Moonjely, S., Bidochka, M.J., Toriello, C., 2019. A review on the genus *Metarhizium* as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico. Biocontrol Sci. Technol. 29, 83–102. https://doi.org/10.1080/09583157.2018.1531111

Canassa, F., Tall, S., Moral, R.A., Lara, I.A.R. d., Delalibera, I., Meyling, N. V., 2019. Effects of bean seed treatment by the entomopathogenic fungi *Metarhizium robertsii* and *Beauveria bassiana* on plant growth, spider mite populations and behavior of predatory mites. Biol. Control 132, 199–208. https://doi.org/10.1016/j.biocontrol.2019.02.003

Chairin, T., Petcharat, V., 2017. Induction of defense responses in longkong fruit (*Aglaia dookkoo* Griff.) against fruit rot fungi by *Metarhizium guizhouense*. Biol. Control 111, 40–44. https://doi.org/10.1016/j.biocontrol.2017.05.012

Clark, R.B., Zeto, S.K., 2000. Mineral acquisition by arbuscular mycorrhizal plants. J. Plant Nutr. 23, 867–902. https://doi.org/10.1080/01904160009382068

David, W.A.L., 1967. The physiology of the insect integument in relation to the invasion of pathogens, in: Beament, J.W.L.; Treherne, J.E. (Ed.), Insects and Physiology. Edinburgh, &c., Oliver & Boyd., pp. 17–35.

Fisher, J.J., Rehner, S.A., Bruck, D.J., 2011. Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. J. Invertebr. Pathol. 106, 289–295. https://doi.org/10.1016/j.jip.2010.11.001

Gange, A.C., Koricheva, J., Currie, A.F., Jaber, L.R., Vidal, S., 2019. Meta-analysis of the role of entomopathogenic and unspecialized fungal endophytes as plant bodyguards. New Phytol. 223, 2002–2010. https://doi.org/10.1111/nph.15859

Gao, Q., Jin, K., Ying, S.H., Zhang, Y., Xiao, G., Shang, Y., Duan, Z., Hu, X., Xie, X.Q., Zhou, G., Peng, G., Luo, Z., Huang, W., Wang, B., Fang, W., Wang, S., Zhong, Y., Ma, L.J., St. Leger, R.J., Zhao, G.P., Pei, Y., Feng, M.G., Xia, Y., Wang, C., 2011. Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet. 7, e1001264. https://doi.org/10.1371/journal.pgen.1001264

Golo, S., Gardner, D.R., Grilley, M.M., Takemoto, J.Y., Krasnoff, S.B., Pires, M.S., Fernandes,

É.K.K., Bittencourt, V.R.E.P., Roberts, D.W., 2014. Production of destruxins from *Metarhizium* spp . fungi in artificial medium and in endophytically colonized cowpea plants. PLoS One 9, e104946. https://doi.org/10.1371/journal.pone.0104946

Gouda, S., Das, G., Sen, S.K., Shin, H.S., Patra, J.K., 2016. Endophytes: A treasure house of bioactive compounds of medicinal importance. Front. Microbiol. 7, 1538. https://doi.org/10.3389/fmicb.2016.01538

Hu, S., Bidochka, M.J., 2021. Abscisic acid implicated in differential plant responses of *Phaseolus vulgaris* during endophytic colonization by *Metarhizium* and pathogenic colonization by *Fusarium*. Sci. Rep. 11, 11327. https://doi.org/10.1038/s41598-021-90232-4

Hu, G., Leger, R.J.S., 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. Appl. Environ. Microbiol. 68, 6383–6387. https://doi.org/10.1128/AEM.68.12.6383

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang, X., Liu, X., Zhan, S., St. Leger, R.J., Wang, C., 2014. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. Proc. Natl. Acad. Sci. U. S. A. 111, 16796–16801. https://doi.org/10.1073/pnas.1412662111

Jaber, L.R., Araj, S.E., 2018. Interactions among endophytic fungal entomopathogens (Ascomycota: Hypocreales), the green peach aphid *Myzus persicae* Sulzer (Homoptera: Aphididae), and the aphid endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae). Biol. Control 116, 53–61. https://doi.org/10.1016/j.biocontrol.2017.04.005

Jaber, L.R., Ownley, B.H., 2018. Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? Biol. Control 116, 36–45. https://doi.org/10.1016/j.biocontrol.2017.01.018

Jallow, M.F.A., Dugassa-Gobena, D., Vidal, S., 2008. Influence of an endophytic fungus on

host plant selection by a polyphagous moth via volatile spectrum changes. Arthropod. Plant. Interact. 2, 53–62. https://doi.org/10.1007/s11829-008-9033-8

Jaramillo, J.L., Montoya, E.C., Benavides, P., Góngora B, C.E., 2015. *Beauveria bassiana* y *Metarhizium anisopliae* para el control de broca del café en frutos del suelo. Rev. Colomb. Entomol. 41, 95–104.

Kepler, R.M., Ugine, T.A., Maul, J.E., Cavigelli, M.A., Rehner, S.A., 2015. Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. Environ. Microbiol. 17, 2791–2804. https://doi.org/10.1111/1462-2920.12778

Keyser, C.A., De Fine Licht, H.H., Steinwender, B.M., Meyling, N. V., 2015. Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. BMC Microbiol. 15, 249. https://doi.org/10.1186/s12866-015-0589-z

Krell, V., Jakobs-Schoenwandt, D., Vidal, S., Patel, A. V, 2018. Cellulase enhances endophytism of encapsulated *Metarhizium brunneum* in potato plants. Fungal Biol. 122, 373–378. https://doi.org/10.1016/j.funbio.2018.03.002

Lacey, L.A., 2012. Manual of techniques in invertebrate pathology, 2nd ed. Elsevier Ltd., Yakima, Washington, EUA.

Lenth, R. V., Buerkner, P., Herve, M., Love, J., Riebl, H., Singmann, H., 2021. Packpage "emmeans."

Liao, X., O'Brien, T.R., Fang, W., St. Leger, R.J., 2014. The plant beneficial effects of *Metarhizium* species correlate with their association with roots. Appl. Microbiol. Biotechnol. 98, 7089–7096. https://doi.org/10.1007/s00253-014-5788-2

Lovett, B., St. Leger, R.J., 2015. Stress is the rule rather than the exception for *Metarhizium*. Curr. Genet. 61, 253–261. https://doi.org/10.1007/s00294-014-0447-9

Mantzoukas, S., Eliopoulos, P.A., 2020. Endophytic entomopathogenic fungi: A valuable

biological control tool against plant pests. Appl. Sci. 10, 360. https://doi.org/10.3390/app10010360

Martins, E.F., Franzin, M.L., Perez, A.L., Schmidt, J.M., Venzon, M., 2021. Is *Ceraeochrysa cubana* a coffee leaf miner predator? Biol. Control 160, 104691. https://doi.org/10.1016/j.biocontrol.2021.104691

McKinnon, A.C., 2016. Plant tissue preparation for the detection of an endophytic fungus in planta. Methods Mol. Biol. 1477, 167–173. https://doi.org/10.1007/978-1-4939-6367-6_13

McKinnon, A.C., Saari, S., Moran-Diez, M.E., Meyling, N. V., Raad, M., Glare, T.R., 2017. *Beauveria bassiana* as an endophyte: a critical review on associated methodology and biocontrol potential. BioControl 62, 1–17. https://doi.org/10.1007/s10526-016-9769-5

Meyling, N. V, Eilenberg, J., 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems : Potential for conservation biological control. Biol. Control 43, 145–155. https://doi.org/https://doi.org/10.1016/j.biocontrol.2007.07.007

Morgan, J.A.W., Bending, G.D., White, P.J., 2005. Biological costs and benefits to plant-microbe interactions in the rhizosphere. J. Exp. Bot. 56, 1729–1739. https://doi.org/10.1093/jxb/eri205

Oliveira, C.M., Auad, A.M., Mendes, S.M., Frizzas, M.R., 2013. Economic impact of exotic insect pests in Brazilian agriculture. Jounal od Appl. Entomol. 137, 1–15. https://doi.org/10.1111/jen.12018

Posada, F., Aime, M.C., Peterson, S.W., Rehner, S.A., Vega, F.E., 2007. Inoculation of coffee plants with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). Mycol. Res. 111, 748–757. https://doi.org/10.1016/j.mycres.2007.03.006

Posada, F., Vega, F.E., 2006. Inoculation and colonization of coffee seedlings (*Coffea arabica*

L.) with the fungal entomopathogen *Beauveria bassiana* (Ascomycota: Hypocreales). Mycoscience 47, 284–289. https://doi.org/10.1007/s10267-006-0308-6

Reis, P.R., Souza, J.C., Venzon, M., 2002. Manejo ecol´ogico das principais pragas do cafeeiro. Inf. Agropec. 23, 83–99.

Reis, P.R., Souza, J.C., 1996. Manejo integrado do bicho-mineiro, *Perileucoptera cofeella* (Guérin-Mèneville) (Lepidoptera: Lyonetiidae) e seus reflexos na produção de café. An. da Soc. Entomológica do Bras. 25, 77-82. https://doi.org/https://doi.org/10.37486/0301-8059.v25i1.1093

Resquín-Romero, G., Garrido-Jurado, I., Delso, C., Ríos-Moreno, A., Quesada-Moraga, E., 2016. Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. J. Invertebr. Pathol. 136, 23–31. https://doi.org/10.1016/j.jip.2016.03.003

Ríos-Moreno, A., Garrido-Jurado, I., Resquín-Romero, G., Arroyo-Manzanares, N., Arce, L., Quesada-Moraga, E., 2016. Destruxin A production by *Metarhizium brunneum* strains during transient endophytic colonisation of *Solanum tuberosum*. Biocontrol Sci. Technol. 26, 1574–1585. https://doi.org/10.1080/09583157.2016.1223274

Roberts, D.W., Hajek, A.E., 1992. Entomopathogenic fungi as bioinsecticides, in: Leatham, F.G. (Eds) Frontiers in industrial micology. Spring, New York, pp.144-159.

Roberts, D.W., 1981. Toxins of entomopathogenic fungi, in: Burges, H.D. (Ed.), Microbial Control of Pests and Plant Disease 1970–1980. Academic Press, London, pp. 441–463.

Sasan, R.K., Bidochka, M.J., 2013. Antagonism of the endophytic insect pathogenic fungus *Metarhizium robertsii* against the bean plant pathogen *Fusarium solani* f. sp. *phaseoli*. Can. J. Plant Pathol. 35, 288–293. https://doi.org/10.1080/07060661.2013.823114

Sasan, R.K., Bidochka, M.J., 2012. The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. Am. J. Bot.

99, 101–107. https://doi.org/10.3732/ajb.1100136

Spatafora, J.W., Sung, G.H., Sung, J.M., Hywel-Jones, N.L., White, J.F., 2007. Phylogenetic evidence for an animal pathogen origin of ergot and the grass endophytes. Mol. Ecol. 16, 1701–1711. https://doi.org/10.1111/j.1365-294X.2007.03225.x

Taiz, L., Zeiger, E., Moller, I.M., Murphy, A. (Eds.), 2017a. Arquitetura da célula e do vegetal, in: Fisiologia e Desenvolvimento Vegetal. Artmed, pp. 1–50.

Tomaz, M.A., Martins, L.D., Rodrigues, W.N., 2015. Produção de mudas e plantio, in: Sakiyama, N.S., Martinez, H.E. Pietro, Tomaz, M.A., Borém, A. (Eds.), Café Arábica: Do Plantio à Colheita. UFV, Viçosa, pp. 46–63.

Ullrich, C.I., Koch, E., Matecki, C., Schafer, J., Burkl, T., Rabenstein, F., Kleespies, R.G., 2017. Detection and growth of endophytic entomopathogenic fungi in dicot crop plants. J. Fur Kult. 69, 291–302. https://doi.org/10.1399/JfK.2017.09.02

Wilson, D., 1995. Endophyte - the evolution of a term, and clarification of its use and definition. Oikos 73, 274–276.

Wyrebek, M., Huber, C., Sasan, R.K., Bidochka, M.J., 2011. Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. Microbiology 157, 2904–2911. https://doi.org/10.1099/mic.0.051102-0

Zimmermann, G., 2007. Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontrol Sci. and Techn., 2007; 17, 879-920. https://doi.org/10.1080/09583150701593963

Zimmermann, G., 1986. The '*Galleria* bait method' for detection of entomopathogenic fungi in soil. J. Appl. Entomol. 102, 213–215. https://doi.org/10.1111/j.1439-0418.1986.tb00912.x

Figures

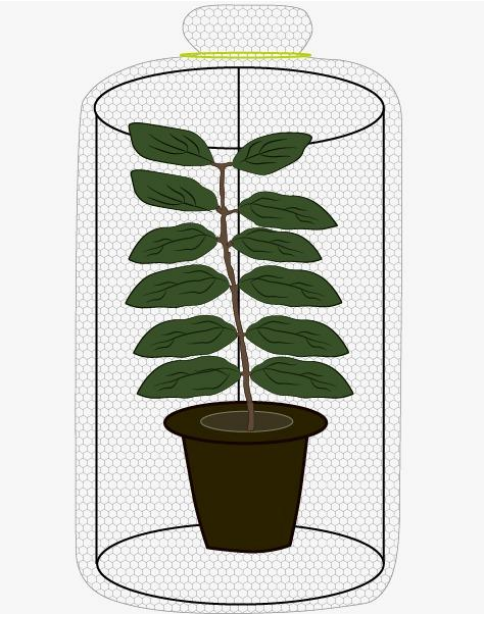

Figure 1 Draft of cylindric cage with coffee seedling

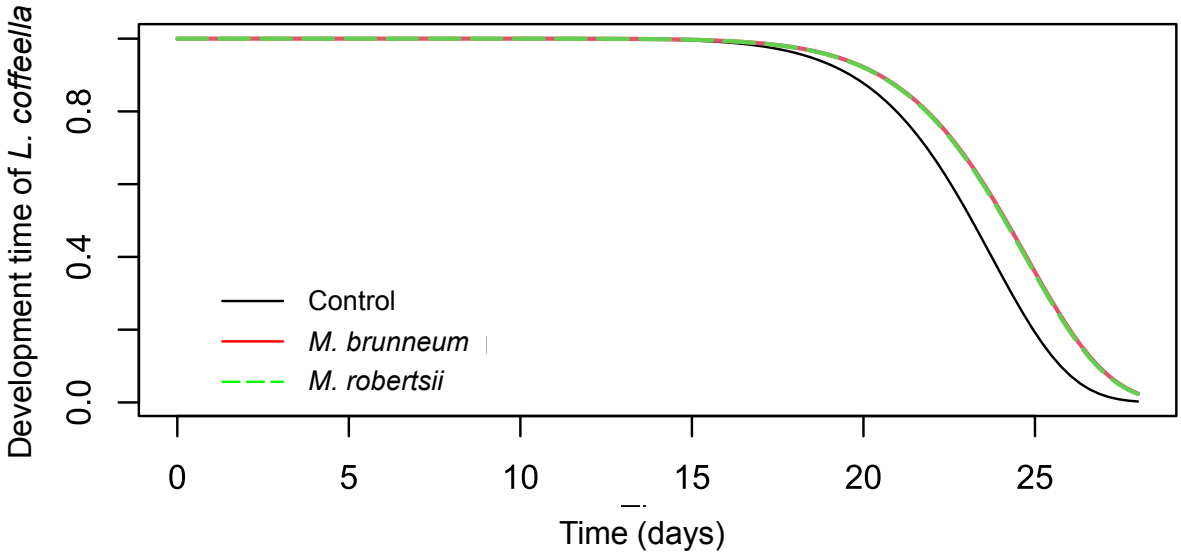

Figure 2 Development time from egg to adult of *L. coffeella* in coffee seedlings with *M. robertsii* and *M. brunneum* inoculation by soil drench. Inoculation of 30 ml of each isolate suspension (10^8) to the surface of the soil in each coffee seedling and control received sterile solution of Tween 0.05 %. Analyses are presented in the text.

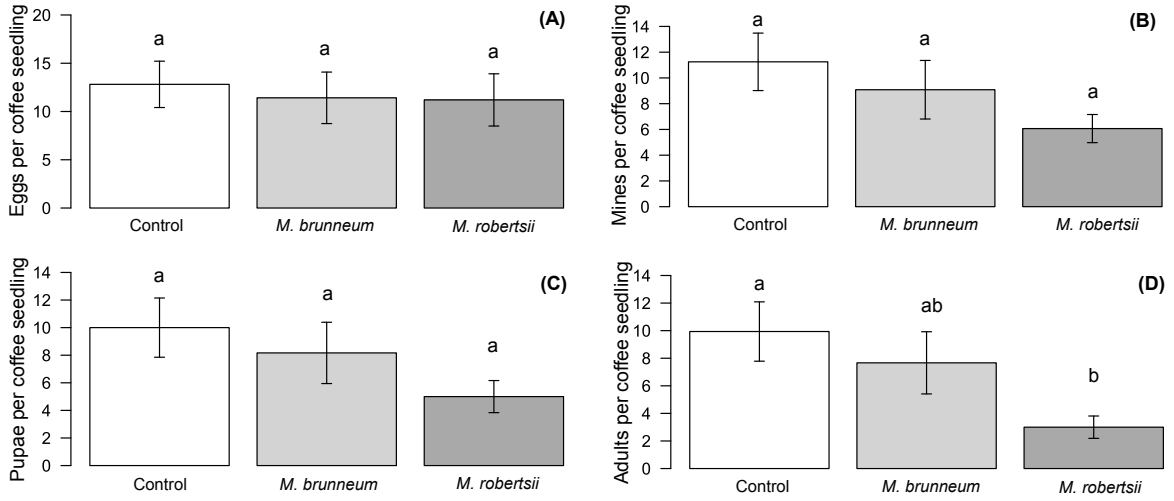

Figure 3 Development of *L. coffeella* in coffee seedlings with *M. robertsii* and *M. brunneum* inoculation by soil drench. (A) number of eggs; (B) number of mines; (C) number of pupae; (D) number of adults. Inoculation of 30 ml of each isolate suspension (10^8) to the surface of the soil in each coffee seedling and control received sterile solution of Tween 0.05 %. Analyses are presented in the text. Bars with same letter have no significant differences.

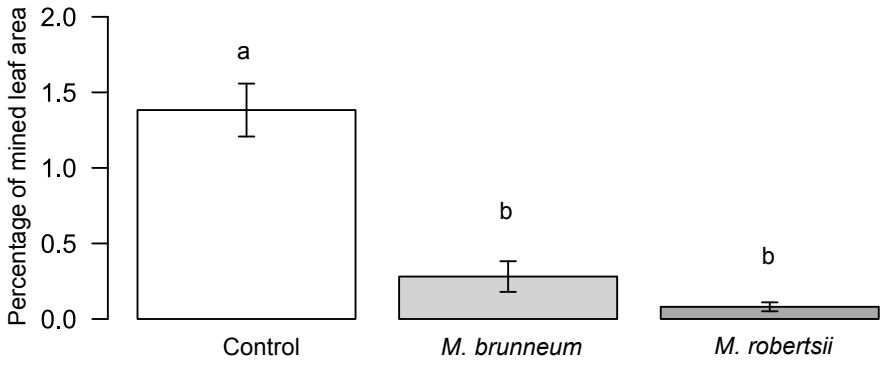

Figure 4 Percentage of mined leaf area by *L. coffeella* in coffee seedlings with *M. robertsii* and *M. brunneum* inoculation by soil drench. Inoculation of 30 ml of each isolate suspension (10^8) to the surface of the soil in each coffee seedling and control received sterile solution of Tween 0.05 %. Analyses are presented in the text. Bars with same letter have no significant differences.

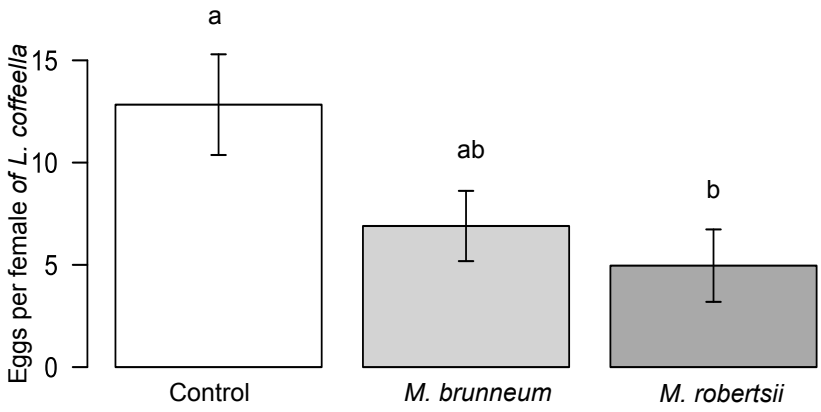

Figure 5 Number of eggs per female of *L. coffeella* emerged in coffee seedlings with *M. robertsii* and *M. brunneum* inoculation by soil drench Inoculation of 30 ml of each isolate suspension (10^8) to the surface of the soil in each coffee seedling and control received sterile solution of Tween 0.05 %. Analyses are presented in the text. Bars with same letter have no significant differences.

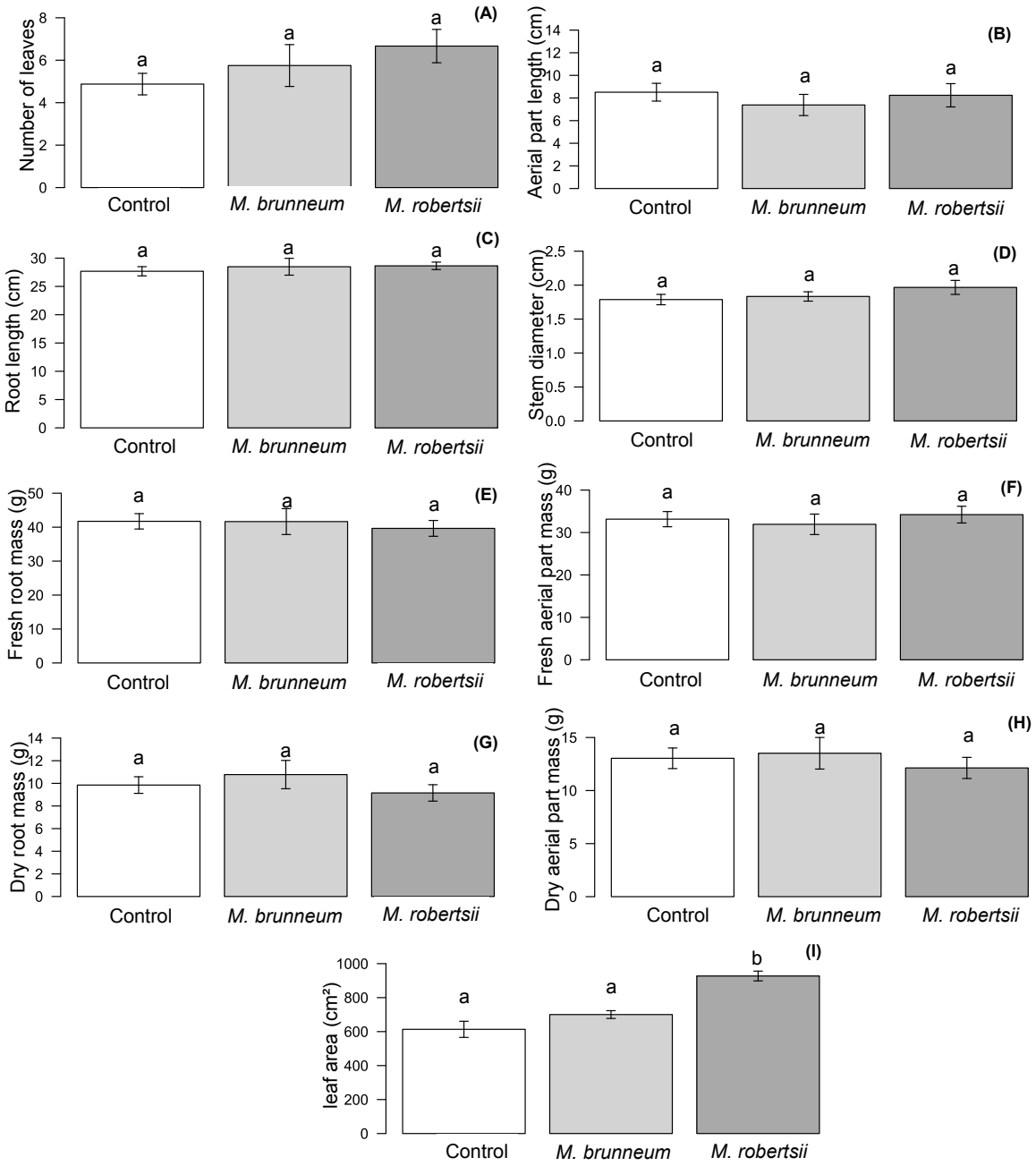

Figure 6 Variables of growth of coffee seedlings with *M. robertsii* and *M. brunneum* inoculation by soil drench. (A) number of leaves; (B) length of aerial part; (C) length of roots; (D) stem diameter; (E) fresh mass of roots; (F) fresh mass of aerial part; (G) dry mass of roots; (H) dry mass of aerial part; (I) leaf area. Inoculation of 30 ml of each isolate suspension (10^8) to the surface of the soil in each coffee seedling and control received sterile solution of Tween 0.05%. Analyses are presented in the text. Bars with same letter have no significant differences.

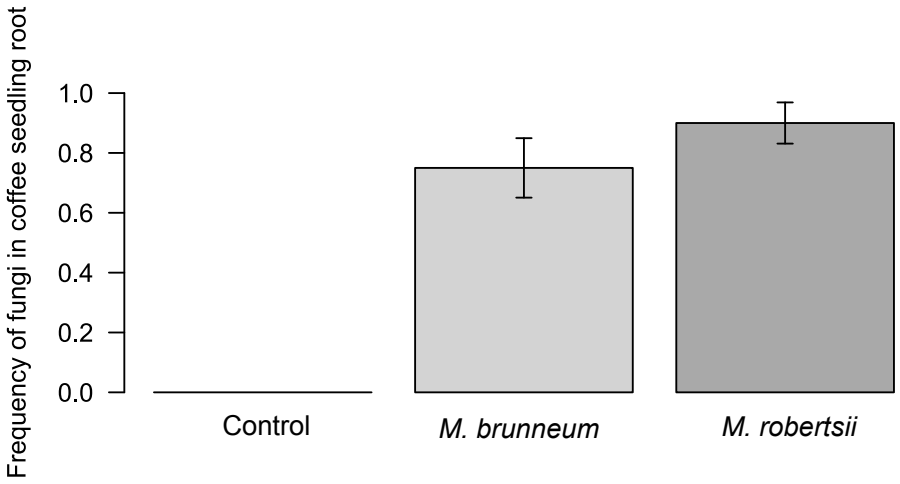

Figure 7 Confirmation of *M. robertsii* and *M. brunneum* coffee roots colonization by soil drench. Inoculation of 30 ml of each isolate suspension (10^8) to the surface of the soil in each coffee seedling and control received sterile solution of Tween 0.05%

Supplementary material

Appendix 1. Agronomic practices in the plots with diversified and conventional coffee systems in the Experimental Research Station of EPAMIG Patrocínio, state of Minas Gerais, Brazil.

Agronomic practices	Date	Diversified coffee system	Conventional coffee system
Soil fertilization	Dec 2018	Urea (750 kg/ha)	Urea (750 kg/ha)
	Feb 2019	NPK 20-05-20 (500 kg/ha)	NPK 20-05-20 (500 kg/ha)
	Oct 2019	Limestone (1.5 t/ha); Gypsum (750 kg/ha); Simple superphosphate (500 kg/ha)	Limestone (1.5 t/ha); Gypsum (750 kg/ha); Simple superphosphate (500 kg/ha)
	Nov 2019	Urea (300 kg/ha)	Urea (300 kg/ha)
	Jan 2020	NPK 20-05-20 (500 kg/ha)	NPK 20-05-20 (500 kg/ha)
	Mar 2020	NPK 20-00-20 (400 kg/ha)	NPK 20-00-20 (400 kg/ha)
	Nov 2020	NPK 20-05-20 (500 kg/ha)	NPK 20-05-20 (500 kg/ha)
	Dez 2020	Simple superphosphate (500 kg/ha)	Simple superphosphate (500 kg/ha)
	Feb 2021	NPK 20-05-20 (500 kg/ha)	NPK 20-05-20 (500 kg/ha)
Coffee husk compost	Dec 2018	5 kg/linear meter	5 kg/linear meter
Foliar fertilization [1]	Dec 2018	Fert Max® (5kg/ha)	Fert Max® (5kg/ha)
	Feb 2019	Fert Max® (5kg/ha)	Fert Max® (5kg/ha)
	Dec 2019	Fert Max® (5kg/ha)	-----
	Feb 2020	-----	Fert Max® (5kg/ha)
	Mar 2020	Fert Max® (5kg/ha)	-----
	Jan 2021	Quimifol Café Cerrado L® (2 l/ha)	Quimifol Café Cerrado L® (2 l/ha)
	Apr 2021	Quimifol Café Cerrado L® (2 l/ha)	Quimifol Café Cerrado L® (2 l/ha)
Insecticide [2]	Feb 2019	-----	Curbix® (2.5 l/ha)
	Feb 2020	-----	Curbix® (2.5 l/ha)
	Jan 2021	-----	Curbix® (2.5 l/ha)
	Apr 2021	-----	Curbix® (2.5 l/ha)
Insecticide /Acaricide [2]	Dec 2018	-----	Batent® (0.4 l/ha)
	Feb 2019	-----	Batent® (0.4 l/ha)
	Feb 2020	-----	Batent® (0.4 l/ha)
	Jan 2021	-----	Abamectin® (0.4 l/ha)
	Apr 2021	-----	Abamectin® (0.4 l/ha)
Fungicide [2]	Dec 2018	-----	Opera® (1.5 l/ha) and Kocide® (1.5 kg/ha)
	Feb 2019	-----	Opera® (1.5 l/ha) and Kocide® (1.5 kg/ha)
	Dec 2019	Kocide® (1.5 kg/ha)	-----
	Feb 2020	-----	Opera® (1.5 l/ha) and Kocide® (1.5 kg/ha)
	Marr 2020	Kocide® (1.5 kg/ha)	-----
	Jan 2021	Kocide® (1.75 kg/ha)	Opera® (1.5 l/ha), Kocide® (1.75 kg/ha) and Cantus (0.15 kg/ha)
	Apr 2021	Kocide® (1.75 kg/ha)	Opera® (1.5 l/ha), Kocide® (1.75 kg/ha) and Cantus (0.15 kg/ha)
Insecticide/Fungicide [2]	Dec 2019	-----	Verdadero® 600 WG (1 kg/ha)
	Dec 2020	-----	Verdadero® 600 WG (1 kg/ha)

Herbicide [2]	May 2019	-----	Zafera® (1 kg/ha) and Aminol® (1 l/ha)
	Nov 2019	-----	Zafera® (1 kg/ha) and Aminol® (1 l/ha)
	May 2020	-----	Zafera® (1 kg/ha) and Aminol® (1 l/ha)
Sodium hypochlorite	Dec 2019	1% Solution	-----
	Mar 2020	1% Solution	-----
	Jan 2021	1% Solution	-----
	Apr 2021	1% Solution	-----
Mechanical suppression of non-crop plants	Jun 2019	Suppressed up to 10 cm	Suppressed up to 10 cm
	Jan 2020	Suppressed up to 50 cm	Suppressed up to 10 cm
	Mar 2020	Suppressed up to 50 cm	Suppressed up to 10 cm
	Jun 2020	Suppressed up to 10 cm	Suppressed up to 10 cm
	Dec 2020	Suppressed up to 50 cm	Suppressed up to 10 cm
	Mar 2021	Suppressed up to 50 cm	Suppressed up to 10 cm
Manual suppression of non-crop plants suppression [3]	Mar 2019	Done	Done
	Apr 2019	Done	Done
	Sep 2019	Done	Done
	Mar 2020	Done	Done
	Apr 2020	Done	Done
	May	Done	Done
Coffee harvest	Jun 2019	Done	Done
	Jun 2020	Done	Done
	May 2021	Done	Done
'Repase' [4]	Oct 2019	Done	Done
	Oct 2020	Done	Done
	Oct 2021	Done	Done

[1] Fert Max ®: potassium (10%), magnesium (2%), sulfur (8.26%), boron (6%), manganese (8%), molybdenum (0.10%) and zinc (3%); Quimifol Café Cerrado L®: nitrogen (10%), boron (1%), manganese (4%), copper (0.5%) and zinc (6%).

[2] Opera® (pyraclostrobin: strobilurin + epoxiconazole: triazole); Verdadero® 600 WG (thiamethoxam: neonicotinoid + cyproconazole: triazole); Batent® (Abamectin: avermectin); Kocide® (copper hydroxide: inorganic); Curbix® (ethiprole: phenylpyrazole); Aminol® (2,4-dichlorophenoxy: aryloxy alkanoic acid); Zafera® (glyphosate: substituted glycine); Abamectin® (Abamectin: avermectin); Cantus® (boscalid: pyridine–carboxamide). All the pesticides were applied with mineral oil as adjuvant Agefix® (0.5%).

[3] Manual suppression uses hoe around the diversified plants and in the flaws in the coffee row.

[4] 'Repase' involves the collection of every coffee berry of all possible stages from the ground, immediately after the harvest.

GENERAL CONCLUSION

The density and activity of *Metarhizium* spp. were higher in diversified coffee system with the species *I. edulis, V. curassavica, S. macranthera* and non-crop plants in the Cerrado of Minas Gerais.

Beta-glucosidase, enzyme involved in organic matter decomposition, has more activity in soil of diversified coffee system. The coffee fruits are weightier in this coffee system. Thus, together with other ecosystem services, such as pollination and biological control by entomophagous, plant diversification has the potential of conserving *Metarhizium* spp. in soil and improving coffee yield.

Metarhizium spp. associates with roots of *I. edulis, V. curassavica, S. macranthera, G. spicatum, C. bonariensis, S. americanum, B. pilosa, A. conyzoides* and *S. cordifolia*. Therefore, these plants improve the maintenance of *Metarhizium* spp. in the coffee crop.

Metarhizium brunneum and *M. robertsii* colonization in coffee roots by soil drench improve growth plant and protection against coffee leaf miner (*L. coffeella*) in coffee seedlings. Therefore, these species have potential as inoculates of coffee seedlings.